The effect of processing on the short- and long-term viability of epididymal African buffalo (*Syncerus caffer*) spermatozoa

by

Maira Margaretha van Leeuwen

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Agricultural Sciences

at

Stellenbosch University

Department of Animal Science, Faculty of AgriSciences

Supervisor: Dr Helet Lambrechts

March 2020

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Summary

The Africa buffalo (*Syncerus caffer*) is one of the Big 5 that features strongly in the ecotourism and trophy hunting industries, and more recently, this species is sought after in game ranching operations. Optimal management of African buffalo in production systems however is hampered by genetic selection for traits without really knowing what the impact on reproduction is, and the diseases African buffalo carry. African buffalo also differ considerably from cattle and even water buffalo when their reproduction is considered. Little information is available on the processing of African buffalo sperm to yield quality samples that can contribute to a genome resource bank of this species, which can then be used for production and conservation purposes (i.e. where entire populations need to be eradicated due to e.g. Foot and Mouth Disease). The study therefore investigated the influence of sperm harvesting method (i.e. processed directly after culling or after 24h of intact storage at 4°C) on the viability of epididymal African buffalo spermatozoa. Spermatozoa aspirated from African buffalo epididymides were evaluated directly after aspiration or subjected to prolonged (i.e. 24h) liquid storage (in Ham's F10) at 5°C to determine the effect of extended liquid storage on the motility, viability, morphology and acrosome integrity of the spermatozoa. Samples that were subjected to 24h of liquid storage post-aspiration were characterized by significantly poorer viability, midpiece abnormalities and total abnormalities. The prolonged intact cold storage of testes had a negative effect on the occurrence of tail abnormalities. Aspirated samples were subjected to cryopreservation in either Triladyl®, or Triladyl® supplemented with trehalose to determine the potential of trehalose supplementation to minimise the deleterious changes caused by cryopreservation. The addition of trehalose had a positive effect on the motility and viability of sperm samples; however, tail morphology was negatively affected. Cryopreserved sperm samples were thawed using two different thawing rates to determine the optimum thawing method to yield samples viable for use in *in vitro* fertilisation procedures or artificial insemination. The thawing rates included a slow thawing rate (37°C for 35 seconds), and a fast thawing rate (80°C for 5 seconds). A fast thawing rate is not recommended due to a significant decrease in the sperm viability. Lastly, flow cytometry was used to determine the potential of this objective analysis method to determine the post-thaw viability of aspirated epididymal African buffalo spermatozoa. Future recommendations include investigation of the influence of prolonged intact storage of testes post-mortem (i.e. up to 72 hours) and at different storage temperatures on epididymal African buffalo sperm viability. Different trehalose supplementation levels and the potential thereof to minimise the negative effect of processing and cryopreservation on spermatozoa warrant further studies. The range of

parameters analysed using flow cytometry should be extended to include parameters such as morphology and acrosome integrity. The influence of extended boma holding stress, as experienced during routine TB monitoring periods, and the influence thereof on sperm viability, and in particular in the presence of elevated lactic acid levels warrants investigation.

Opsomming

Die Afrika-buffel (Syncerus caffer) is een van die Groot 5 wat ’n gesogte spesie in die ekotoerisme- en trofeejagbedryf is en wat meer onlangs as gesog in wildboerderybedrywighede beskou word. Die optimale bestuur van Afrika-buffels in produksiestelsels word egter belemmer deur die genetiese seleksie vir eienskappe sonder om regtig te weet wat die impak op voortplanting is, en die siektes wat Afrika-buffels dra. Afrika-buffels verskil ook aansienlik van beeste en selfs waterbuffels as hulle voortplanting oorweeg word. Daar is min inligting beskikbaar oor die verwerking van Afrika-buffelsperm om kwaliteitmonsters te lewer wat kan bydra tot 'n genoomhulpbronbank van hierdie spesie, wat dan vir produksie- en bewaringsdoeleindes gebruik kan word (d.w.s. waar die hele bevolking uitgeroei moet word as gevolg van Bek-en-klouseer). Die studie het gevolglik die invloed van die oesmetode (d.w.s. direk verwerk na uitdunnning of na 24 uur van intakte berging by 4°C) op die lewensvatbaarheid van die epididimale Afrika-buffelsperme ondersoek. Sperme wat deur middel van aspirasie uit Afrika-buffel epididimii versamel is, is direk na aspirasie geëvalueer of aan langdurige (d.w.s. 24 uur) berging in Ham's F10 by 5°C beoordeel om die effek van langdurige berging op die beweeglikheid, lewensvatbaarheid, morfologie en akrosoom integriteit van die sperme te bepaal. Monsters wat aan 24 uur van berging na aspirasie onderworpe was, is gekenmerk deur aansienlik swakker lewensvatbaarheid, middelstuk abnormaliteite en totale abnormaliteite. Die langdurige intakte berging van testes het 'n negatiewe uitwerking op die voorkoms van stert abnormaliteite gehad. Aspireerde monsters is in óf Triladyl®, óf Triladyl® aangevul met trehalose gevries om die potensiaal van trehalose-aanvulling te bepaal om die nadelige veranderinge wat veroorsaak word deur diepbevriesing, te minimaliseer. Die toevoeging van trehalose het 'n positiewe invloed op die beweeglikheid en lewensvatbaarheid van spermmonsters gehad; die stertmorfologie is egter negatief beïnvloed. Die bevrore spermmonsters is teen twee verskillende ontdooiingtempo’s ontdooi om die optimale ontdooiingsmetode te bepaal om monsters lewensvatbaar te maak vir gebruik in in vitro bevrugtingsprosedures of kunsmatige inseminasie. Die ontdooiingstempo’s het 'n stadige ontdooiingstempo (37°C vir 35 sekondes) en 'n vinnige ontdooiingstempo (80°C vir 5 sekondes) ingesluit. 'n Vinnige ontdooiingstempo word nie aanbeveel nie as gevolg van 'n beduidende afname in die lewensvatbaarheid van die sperme. Laastens is die potensiaal van vloeisitometrie om as metode gebruik te word om die lewensvatbaarheid van die bevrore-ontdooide epididimale Afrika buffelsperme te bepaal, ondersoek. Toekomstige aanbevelings sluit in die ondersoek na die invloed van langdurige intakte berging van testes na uitdunning (dit wil sê tot 72 uur) en by verskillende bergingstemperature op die

lewensvatbaarheid van die epididimale Afrika buffelsperme. Verskillende trehalose aanvullingsvlakke en die potensiaal daarvan om die negatiewe effek van prosessering en diepbevriesing op sperme tot die minimum te beperk, is 'n verdere ondersoek. Die reeks parameters wat met behulp van vloeisitometrie ontleed word, moet uitgebrei word om parameters soos morfologie en akrosoom integriteit in te sluit. Die invloed van langdurige aanhou in bomas, soos ondervind tydens roetine TB-moniteringstydperke en die invloed daarvan op die lewensvatbaarheid van sperme, veral in die teenwoordigheid van 'n verhoogde melksuurvlak, moet ook ondersoek word.

Acknowledgements

Firstly, I would like to thank Dr Helet Lambrechts for her support and encouragement, without whom I would not have managed to get through these last 2 years.

Another big thank you goes to Gail Jordaan for the statistical support needed during my data analysis and for showing me the fun side to statistics.

Further, I would like to thank the team at Hluhluwe-iMfolozi Game Reserve who made this trial possible. Your help, advice and sense of humour will never be forgotten!

To Kirsty, Francesca, Michal, Nastasia and Dr Esposito, you all were my support structure throughout the trial and without you I surely would have had a few breakdowns. I am forever grateful for your positivity, humour and cooking skills.

Thank you to the Department of Animal Sciences of Stellenbosch University and the Stellenbosch University Post-graduate office for helping to fund my first year of study.

Thank you to all of my friends who have given me a listening ear and always been handy with advice should it be needed. You know who you are and I appreciate you all so much.

Thank you to the Trollip family, I am eternally grateful for your unwavering support.

Thank you to Chad for the constant support end never-ending encouragement, I cannot express my gratitude enough.

Lastly, thank you to my amazing parents for the emotional and financial support through these times. To my mom, your immense sacrifice to allow me to follow my dreams is the greatest gift of all, I love you infinitely.

Preface

This thesis is presented as a compilation of 7 chapters.

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Alphabetical List of Abbreviations

°C	Degrees Celsius
µL	Microliters
Abn.	abnormal
AI	Artificial Insemination
ART	Assisted Reproductive Techniques
ATP	Adenosine triphosphate
C	Concentration
cc	Cubic centimetre
DAFF	Department of Agriculture and Fisheries
DNA	Deoxyribonucleic acid
ET	Embryo transfer
FMD	Foot-and-mouth disease
G	Gauge
GDP	Gross Domestic Product
GRB	Genome Resource Bank
h	Hours
H_2O	Water
ID	Identity
IUCN	International Union for Conservation of Nature
IVEP	*In Vitro* Embryo Production
kg	Kilogram

LN_2	Liquid Nitrogen
m	Mass
M	Molar
mM	milliMolar
N	Number of samples/animals
No.	Number
PVC	Polyvinyl Chloride
ZAR	Rand
SD	Standard deviation
T+T	Triladyl® supplemented with trehalose
TB	Tuberculosis
Tri	Triladyl®
V	Volume

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Chapter 1

General Introduction

The South African wildlife industry is structured around four main components, namely trophy hunting, eco-tourism, the breeding of game as well as the production of game products such as meat, hides as well as other souvenirs such as jewellery. The wildlife industry as a whole contributes significantly to the South African economy with game and trophy hunting contributing approximately 7.3 billion ZAR to the Gross Domestic Product (GDP) of South Africa. The game ranching industry, which consists of privately owned farms, is expanding rapidly, as evident when considering the number of game animals which exceed that of cattle in South Africa (Oberem, 2015).

The African buffalo (*Syncerus caffer*) is a member of the Family Bovidae and being a member of Africa's Big 5, is also a preferred species to maintain in terms of ecotourism and trophy hunting activities (Coleman, 2018). The African buffalo contributed 12% to South Africa's trophy hunting earnings in 2015, with the total annual foreign currency trophy hunting earnings of 2015 equalling to 1.2 billion ZAR (Coleman, 2018). African buffaloes have an average live weight of 590kg, with some reported to weigh up to 1000kg. The African buffalo also plays a crucial role within an ecosystem with regards to the conversion of long grassland to short grassland to allow smaller herbivores to utilise the veld more efficiently (Michel & Bengis, 2012, Krugerpark.co.za, 2017). Being a preferred species in ecotourism and trophy hunting operations, there has in recent years been a demand for high genetic merit breeding animals, and specifically for males with a large horn span (Smith, 2015; Coleman, 2018). South Africa has approximately 2500 registered African Buffalo breeders with the disease-free herd population ranging between 50 000 to 70 000 individuals (Coleman, 2018).

As of February 2018, the African buffalo has been listed as a Near Threatened species, with wild population estimates being approximately 400 000 and this population is facing a decreasing trend (IUCN Red List, 2019). This decreasing trend is not only due to anthropogenic factors but also due to the disease susceptibility of the African buffalo. Foot-and-mouth disease (FMD), Bovine Tuberculosis, brucellosis as well as corridor disease all affect the African buffalo populations, with FMD as well as bovine tuberculosis having the most pronounced negative effect on the economy. Foot and mouth disease, which is caused by the *Aphthovirus*, can be transferred to domestic cattle, which is why there are restrictions on the movement and transport of African buffalo (Laubscher & Hoffman, 2012; Jori *et al*., 2016; Perumal *et al*.; 2016). It has been found that FMD results in a reduction in an animal's

fertility thus causing potential income loss to commercial farmers (Chaters *et al*., 2018). Bovine tuberculosis, which is caused by *Mycobacterium spp.*, is not only transferrable to domestic cattle but also poses a zoonotic risk (Michel *et al.* 2006; DAFF, 2016). This disease is highly contagious and can lead to the condemnation of infected carcasses, infection of milk as well as a decline in the reproductive efficiency of affected animals (Michel *et al*., 2006; DAFF, 2016; Perumal *et al*., 2016). The African buffalo is considered a maintenance host of *Mycobacterium bovis* which means that the infection remains within the population even without external re-infection occurring (Renwick *et al*., 2007).

Genome resource banking (GRB) is defined as "the storage of gametes (ova and spermatozoa) and embryos from threatened populations, with a deliberate intention to use them in a breeding program at some future occasion." The human race can therefore aid in preserving or allowing for the re-introduction of species by cryopreserving the genetic material in liquid nitrogen at a temperature of -196°C (Sezarc.org, 2014). This method not only allows for national exchange of genetics but also extends to the international exchange of genetic material to aid in conservation of species that are at risk. Due to these reasons as well as the African Buffalo being affected by various diseases it is becoming crucial to research ways in which we can use Assisted Reproductive Techniques (ART's) to harvest the spermatozoa from African buffalo bulls to aid in the production of offspring as well as to create stock of African buffalo genetics in the Genome Resource Bank.

Assisted reproductive techniques (ARTs) form an integral component of the establishment and maintenance of genome resource banks. The collection or harvesting, evaluation, processing and storage of spermatozoa and oocytes of wildlife species requires the development of species-specific protocols to ensure gamete viability and fertilising ability are maintained. Short-term storage of sperm samples in a liquid state allows for spermatozoa to be stored with minimal processing to be used for procedures such as artificial insemination or *in vitro* embryo production. Short-term storage eliminates the need for the cryostorage of samples, thus making it a less costly procedure when compared to long-term storage such as cryopreservation using liquid nitrogen (Raseona *et al*., 2017). Cryopreservation is the method used for the long and indefinite storage of spermatozoa however; cryodiluents have to be used in order to protect the spermatozoa from events such as ice crystal formation, cell membrane damage, cold shock and to supply nutrients to the spermatozoa. Various commercial cryodiluents are available, with Triladyl® considered the recommended cryodiluent for African buffalo spermatozoa (Lambrechts *et al*., 1999; Herold *et al*., 2004). Egg yolk is one of the main ingredients of Triladyl®. Egg yolk is not only rich in nutrients, but it is believed that the egg yolk in the cryodiluent protects spermatozoa from cold shock incurred during cryopreservation (Moussa *et al*., 2002; Bergeron *et al*., 2004). Supplements,

such as trehalose or glycerol, can be added to a cryodiluent in order to provide either a nutritive and/or a protective role to spermatozoa during cryopreservation. Trehalose is a disaccharide that provides energy to the spermatozoa during equilibration, cryopreservation and thawing, with the main function being the maintenance of the osmotic balance of the cryodiluent mixture that in turn will minimise the potential cell membrane damage that can occur during cryopreservation. Trehalose is also responsible for the reduction of the formation of ice crystals within spermatozoa during the cryopreservation process, with the latter that can most often result in cell damage that will ultimately reduce the viability and fertilising ability of spermatozoa (Shaikh *et al*., 2016; Zhu *et al*., 2017; Iqbal e*t al.,* 2018).

When previous studies on the development of an African buffalo specific protocol for the long-term storage of African buffalo spermatozoa are considered, the optimum equilibration time as well as type of cryodiluent have been investigated. In a study conducted by Herold *et al.* (2004) it was found that equilibration times ranging between 4 to 9 hours had no influence on the post-thaw sperm quality, however shorter equilibration times were detrimental to the spermatozoa. Their study also found that Triladyl® was the preferred cryodiluent when compared to AndroMed® and Red Ovine Freezing buffer, supporting the findings of Lambrechts *et al.* (1999) where Triladyl® yielded better results than sperm-TALP. Initially the assumption that degenerative testis tissue, resulting from prolonged storage, may have a detrimental effect on spermatozoa was followed (Hopkins *et al.*, 1988; Herold *et al*. 2004) however, in a later study conducted by Herold *et al.* (2006) the storage of intact African buffalo epididymides for up to nine hours at 4°C indicated that viable spermatozoa could be harvested from the epididymides.

The study therefore aimed to determine the influence of different pre-cryopreservation processing protocols (i.e. extended cold storage (24h at 5°C) of intact testes, and extended storage (24h at 5°C) of aspirated epididymal African buffalo spermatozoa) on the viability, motility, morphology and acrosome integrity of African buffalo spermatozoa. The potential of trehalose to minimise the deleterious changes that can occur during pre-thaw and post-thaw processing, was also investigated. Cryopreserved samples were subjected to two different thawing rates to determine whether the potential protective effect of trehalose carry over to post-thaw viability, motility, acrosome integrity and morphology of epididymal African buffalo spermatozoa. The potential of flow cytometry to quantify post-thaw epididymal African buffalo sperm viability was also investigated. Findings from this study will contribute meaningfully to the refinement of protocols for the harvesting, processing and cryopreservation of genetic material from high value genetics of African buffalo bulls, which can be used for research on or propagation and thus conservation of this species.

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Chapter 2

Literature Review

2.1 Introduction

The South African wildlife industry is structured on four pillars, namely trophy hunting, eco-tourism, the breeding of game as well as the production of game products. These four pillars contribute more than 20 billion ZAR to the GDP of the country annually, with hunting generating the most income. Hunting activities and sales of the animals also play a vital role in terms of food security. The local game ranching industry is expanding at a rapid rate and as of 2015, there were 20 million head of game on private ranches within South Africa, compared to only 14 million head of cattle during the same time period (Oberem, 2015).

The African buffalo (*Syncerus caffer*) is a large African herbivore that can reach a live weight of 950 kg and occupies an important position in the ecosystems they are found in. Buffaloes are bulk grazers and thus allow for the conversion of long grasslands into environments characterised by short grasslands, which in turn opens the landscape for smaller herbivores such as the impala (*Aepyceros melampus*) that exhibit more selective feeding habits (Michel & Bengis, 2012, Krugerpark.co.za, 2017). The African buffalo thus play an important role in the maintenance of the quality of the available grazing by digesting the long, fibrous grasses that the smaller herbivores have difficulty feeding on.

The African buffalo is a member of the so-called Big 5 and is therefore highly sought after in eco-tourism and trophy hunting operations. According to the International Ecotourism Society (2015), ecotourism is defined as the responsible travel to natural areas that conserves the environment, sustains the well-being of the local people, and involves interpretation and education of both staff as well as guests. Trophy hunting is the act of paying for and hunting of an animal for its meat as well as the “trophy”, with the latter that may include the hide and/or the head. Trophy hunting provides much needed funding for the conservation of threatened individuals as well as have a positive impact on the local economy. As of October 2018, there were approximately 2500 registered African buffalo farms in South Africa, with privately owned disease-free herds of African buffalo amounting to between 50 000 – 70 000 animals (Coleman, 2018). In 2015, the African buffalo contributed R145 million to South Africa’s annual foreign currency exchange (i.e. total of 1.2 billion ZAR), which represents 12% of the total earnings from hunting activities for that year, coming in second to lion hunting (Coleman, 2018). Revenue obtained from trophy hunting is also necessary to justify the large amount of land that protected wildlife areas occupy which

is becoming an even bigger concern with the continuous need and increased demand for land and more space for infrastructure such as housing and farming (Cruise, 2016).

Unfortunately due to trophy hunting; unintended selection against big horn span has occurred in the industry. The game ranching of buffalo is becoming increasingly popular, becoming a multi-million dollar industry, with the main goal being to select and breed genetics with a wide horn span (Smith, 2015; Coleman, 2018). One approach to overcome the difficulty of farming with African buffalo, is to make use of genome resource banks that can provide national and international producers access to the germplasm of high genetic merit African buffalo cows and bulls. A set of protocols that will allow for the collection, storage and transport of African buffalo sperm can open up the possibility of breeding programs, and also allow for the conservation of genetic material in the case of epidemic outbreaks of diseases that might threaten the existence of this indigenous wildlife species. With a successful method of sperm harvesting, storage and transportation genetic exchange can occur on an international level either with zoo's or conservation programs in other African countries such as Namibia and Botswana, without having to transport the African buffalo bulls across the border and thus limiting the spread of endemic diseases into other countries. This exchange of genetic material will potentially contribute to the maintenance of the genetic diversity of the species, thus decreasing the risk of inbreeding and the negative effects associated with it.

2.2 African buffalo as a production species

The African buffalo has a lifespan of up to twenty five years. African buffalo can weigh close to one tonne, with the average range being between 550-900 kg.Females occupy the lower portion of this range, and males the higher portion. The African buffalo species is distributed across Sub-Saharan Africa, but is mostly found in game reserves, where they live in large, mixed sex herds in the Savannah grassland, often near river beds or mud holes (African Wildlife Foundation, not dated.; Bradford, 2014; Hluhluwe Game Reserve, 2019). The age at which an African buffalo enters puberty is dependent on their live weight. When females attain a live weight of approximately 350 kg (age of just over three years) they enter puberty, and in most cases females will also start ovulating then. However, African buffalo cows are only considered sexually mature at an age of five years, when they will have their first calf. Spermatogenesis in African buffalo bulls can start as early as at two and a half years of age, however, bulls often only start contributing to the genetic gene pool at an age of 7 to 8 years (Cromhout, 2014).

The length of the oestrus cycle in sexually mature African buffalo cows is approximately 23 days, and oestrus lasts between 5 and 6 days (Furstenburg, 2018). African buffaloes have a gestation period of 340 days (i.e. approximately 11 months) and usually give birth to one live offspring during the rainy season to ensure that there is adequate nutrition available for both the dam and calf to cope with the physiologically demanding activities of lactation and growth, respecitively. The long gestation period results in African buffalo cows falling pregnant only every other year due to not coming into oestrus soon after parturition, which allows the animals to replenish their reserves and thus prepare for the next gestation period (Furstenburg, 2010). Optimal management of African buffalo in production systems is of vital importance to ensure the maintenance of captive and free-roaming populations (African Wildlife Foundation, not dated.; Bradford, 2014; Hluhluwe Game Reserve, 2019).

2.2.1 Disease susceptibility of the African buffalo

The African buffalo is particularly susceptible to four diseases, namely foot-and-mouth disease (FMD), Corridor disease, Bovine Tuberculosis and Brucellosis (Laubscher & Hoffman, 2012). These diseases affect various livestock species such as cattle (*Bos taurus*) (Tanner *et al*., 2014; Knight-Jones *et al*., 2016), pigs (*Sus scrofa domesticus*), goats (*Capra aegagrus hircus*) (DAFF, 2016; Knight-Jones *et al*., 2016) and sheep (*Ovis aries*) (Knight-Jones *et al.*, 2016). These diseases have been reported in African buffalo herds of the Kruger National Park in Mpumalanga, and the Hluhluwe-iMfolozi Park in Kwa-Zulu Natal (De Vos & Van Niekerk, 1969; Laubscher & Hoffman, 2012).

Foot and Mouth Disease (FMD) is an infectious disease caused by an *Aphthovirus* and the disease has a low mortality rate. Infections can occur at an age of around 7 months, and can be dormant in the animal (i.e. not showing any physical signs of infections) for a number of years, even after recovery from an acute infection (Ayebazibwe *et al.,* 2010; Laubscher & Hoffman, 2012).The effect this disease has on fertility and breeding thus needs to be taken into account in the formulation of management plans and breeding programs. Foot and mouth disease can be transmitted between African buffalo through the use of cryopreserved spermatozoa, and also from African buffalo to domestic cattle through close contact. Due to this disease being highly contagious it is preferred not to transport African buffalo to areas of livestock production (Laubscher *&* Hoffman*,* 2012; Jori *et al*., 2016; Perumal et. al., 2016).

Corridor disease is caused by *Theileria parva* which is transmitted by infected ticks, and it is a disease that affects cattle as well as buffalo. The ticks become infected by parasitizing on infected African buffaloes, and consequently transmit it to domestic cattle upon attachment and feeding. This disease not only poses an economic risk but it also result in acute fatalities in infected cattle (Laubscher *&* Hoffman*,* 2012, Mitchell, 2018). Due to this reason, not a lot

of research has been conducted considering the effects on fertility and breeding due to very few animals surviving the disease once they have been infected (Laubscher & Hoffman, 2012).

Bovine tuberculosis is caused by *Mycobacterium* species, with the most common bacteria that causes infection in African buffalo being *Mycobacterium bovis*. *Mycobacterium bovis* is a zoonotic pathogen which results in extra emphasis being placed on the reduction of occurrences as well as the potential eradication of the disease (Michel *et al.*, 2006; DAFF, 2016). The disease is spread via infected body tissues and milk, as well as contact of infected mucus with water and feed (roughage or grazing). The disease results in various losses such as decreased profits due to having to condemn infected carcasses, unable to sell infected milk, loss of offspring from infected individuals as well as a considerable decline in the reproductive efficiency of infected animals (Michel *et al.*, 2006; DAFF, 2016; Perumal *et al*., 2016). The disease has European origins through the importation of European cattle breeds, and was first recorded in South Africa in the late 19th century, whereafter an increase in incidence resulted as production systems became increasingly more intensive in nature. The African buffalo is considered a maintenance host of *Mycobacterium bovis*, which means that the infection remains within the population even without external reinfection occurring (Renwick *et al*., 2007).

Brucellosis, which is also known as contagious abortion in cattle and African buffalo, is a zoonotic disease which is caused by *Brucella abortus* and *Brucella melitensis* (Perumal *et al.*, 2016). The bacteria can be transmitted orally via the placenta, vaginal discharge, mammary secretions, and breeding with infected sperm or animals should be avoided (Laubscher & Hoffman, 2012; Ducrotoy *et al*., 2017). The disease causes the female animal to abort in the last trimester, thus resulting in huge economic losses, which is why regulating the disease is becoming increasingly important (Laubscher & Hoffman, 2012; Gorsich *et al*., 2015; Ducrotoy *et al*., 2017).

The incidence and impact of the abovementioned diseases on the production and reproduction of African buffalo warrants the development of collection and processing protocols to allow for the harvesting and long-term storage of African buffalo sperm and oocytes that can contribute to a genome resource bank for the species. Genetic material can thus be accessible for *ex situ* conservation should the species be threatened with extinction and development of such protocols opens up the possibility to exchange genetic material between breeding populations nationally and internationally.

2.2.2 Role of genome resource banks and ARTs in African buffalo production

Genome resource banking (GRB) is defined as “the storage of gametes (ova and spermatozoa) and embryos from threatened populations with a deliberate intention to use them in a breeding program at some future occasion” (Sezarc.org, 2014). The human race can therefore aid in preserving or allowing for the reintroduction of species by cryopreserving the genetic material in liquid nitrogen at a temperature of -196°C. Genome resource banking is most commonly associated with the collection of spermatozoa from harvested testes of a deceased animal, however it can also include urine samples, blood samples and tissue samples, as well as a number of other biological samples (Sezarc.org, 2014). According to the IUCN (International Union for Conservation of Nature) Red List there are currently (as of 2018) only about 400 000 wild, mature African buffalo and the conservation status of this species is listed as Near Threatened. With this trend in decreasing numbers, the importance of establishing new methods for gamete harvesting and storage is thus becoming increasingly important.

Artificial reproductive techniques (ARTs) is the collective name given to the collection, handling and storage of human and/or animal gametes (male or female) to assist in the artificial reproduction of a species. Artificial reproductive techniques include amongst others, semen collection from a male, artificial insemination (AI) and *in vitro* embryo production (IVEP), with all these techniques having the collective goal of improving the reproductive efficiency of a species. In order to use ARTs successfully, knowledge of both the male and female reproductive tract as well as how their gametes (oocytes and sperm) need to be harvested, stored and used, is essential.

The use of ARTs in the livestock industry is becoming increasingly popular due to the fact that the reproduction of the species being farmed with, plays a crucial role in determining the viability and sustainability of a production system. The use of ARTs to optimise *ex situ* African buffalo production systems has been studied by a handful of authors, i.e. Lambrechts (1996), Herold *et al*. (2004) and Herold *et al*. (2006). The three most applied ARTs that may find application in African buffalo production systems, include AI, IVEP and embryo transfer (ET).

Artificial Insemination (AI)

Artificial Insemination (AI) is the process by which spermatozoa are introduced artificially into the cervix or uterus to facilitate fertilisation in ways other than mating (Hafez & Hafez, 2008). This method is the most applied in livestock reproduction in order to introduce new genetics

in a breeding herd or flock, to prevent injury to the female animal or staff (due to not needing a male animal), to synchronise the insemination and conception for multiple animals as well as for more accurate genetic and management record keeping. Other advantages include making local herd/flocks genetics available internationally, thus eliminating the need to transport male animals in order to sire offspring as well as eliminating the need for herds/flocks to have breeding males present (Dairy Mail Africa, 2009). A few disadvantages of AI are that staff needs to be trained how to perform AI as well as how to successfully detect heat in a female to ensure the optimal time of insemination to ensure successful fertilisation after insemination has been carried out.

The main semen collection methods for AI purposes include the artificial vagina method and electro-ejaculation. Semen samples collected using these two methods can either be used fresh, stored in liquid form at 4-5°C for up to 72 hours (depending on species) or cryopreserved for later use. Before short- or long-term storage, semen samples used for AI need to be subjected to macroscopic and microscopic evaluation. Macroscopic parameters include mass motility, colour, pH and viscosity (Hafez & Hafez, 2008). Microscopic parameters include motility, concentration, morphology, morphometry and acrosome integrity.

In cattle or other bovid species a recto-vaginal insemination method is often used. With this method, one arm is inserted into the rectum whilst the other hand guides the pistolet (containing the semen straw) into the vagina. The rectal hand holds and stabilises the cervix in order for the pistolet to be gently manoeuvred through the cervix and into the uterus for insemination (Afimilk, 2015). Artificial insemination in water buffaloes (*Bubalus bubalis*) has been taking place since the 1950's, with procedures similar to those used in domestic cattle. A limiting factor regarding successful insemination of buffaloes is an obscured expression of oestrus, which in turn result in difficulty to establish the optimum window period for insemination (Vale *et al*., 2014). According to Bernard Wooding (Ferreira, 2013) it is possible to artificially inseminate African buffalo. However, due to the temperament of these animals it can be a dangerous procedure. Insemination of African buffalo resulted in a conception rate of 50%, thus the risk to perform this procedure is not justified when the higher conception rate after natural mating (i.e. approaching 100%) is considered (Ferreira, 2013).

In-vitro embryo production

In vitro production of embryos involves the collection of oocytes from live donors by means of flushing or transvaginal ultrasound-guided aspiration after superovulation, or by harvesting from the ovaries of a culled or deceased female. The oocytes are allowed to mature for a

period of 24 hours under controlled conditions, and then fertilised with hyperactivated spermatozoa. Prior to the introduction of sperm to matured oocytes, a gradient test is often performed to separate viable sperm from non-viable sperm in order to ensure that only viable sperm is introduced to the oocyte (Hansen, 2017). The viable sperm also needs to undergo capacitation before it is able to penetrate the oocyte's cumulus cells and ultimately fertilise the oocyte. Capacitation is most commonly brought about by introducing heparin to the Petri dish containing the oocyte and viable sperm (Rehman *et al.,* 2001). A prime example of *in vitro* embryo production in the African buffalo is the birth of Pumelelo in 2016 that resulted from the fertilisation of an oocyte that was collected by means of transvaginal ultrsound-guided ovum pick-up from an African buffalo cow with frozen-thawed African buffalo spermatozoa (Embryo Plus, 2016).

Owiny *et al*. (2009) attempted to produce cattle/African buffalo hybrid embryo's in order to produce offspring that are more resistant to diseases such as East Coast Fever, and to allow for domestication of cattle/African buffalo hybrids. Unfortunately the hybrid embryos did not develop past the morula stage.

Embryo transfer

Embryo transfer is the process by which one or more embryos produced naturally are flushed from high genetic merit donor cows. Alternatively embryos can be obtained artificially using an *in vitro* approach, with resulting embryos than transferred to synchronized recipient cows. Embryo transfer allows for accelerated genetic improvement due to the production of multiple offspring from the same high genetic merit cow as opposed to only one calf born from natural pregnancy (Troxel, not dated.). As mentioned above, the first successful case of IVF and subsequent embryo transfer occurred in 2016 at the Lekkerleef Buffalo ranch, which resulted in the birth of Pumelelo, with a bovine cow that was used as a surrogate (Embryo Plus, 2016).

All the above ARTs can be used to ensure the establishment of disease-free African buffalo herds, with the oocytes from disease-free parent stock being utilised as the basis for the establishment of the disease-free herds. The use of the three ART's prevent the spread of diseases, allow for the national and international distribution of genetics from the top genetic merit African buffaloes, thus contributing to the maintenance of the genetic diversity of this species.

2.3 African buffalo bull reproduction

2.3.1 Anatomy of reproductive organs

The reproductive organs of a male animal are responsible for the production, maturation, and transportation of spermatozoa, as well as supplying the components to keep the spermatozoa viable and able to successfully fertilise an ova. The anatomy of the male reproductive system can be divided into three main components, namely the testes, the accessory glands (consisting of the bulbo-urethral gland, seminal vesicles, prostate and ampulla), and the secondary sex organs that include the penis, epididymis and vas deferens (Figure 2.1).

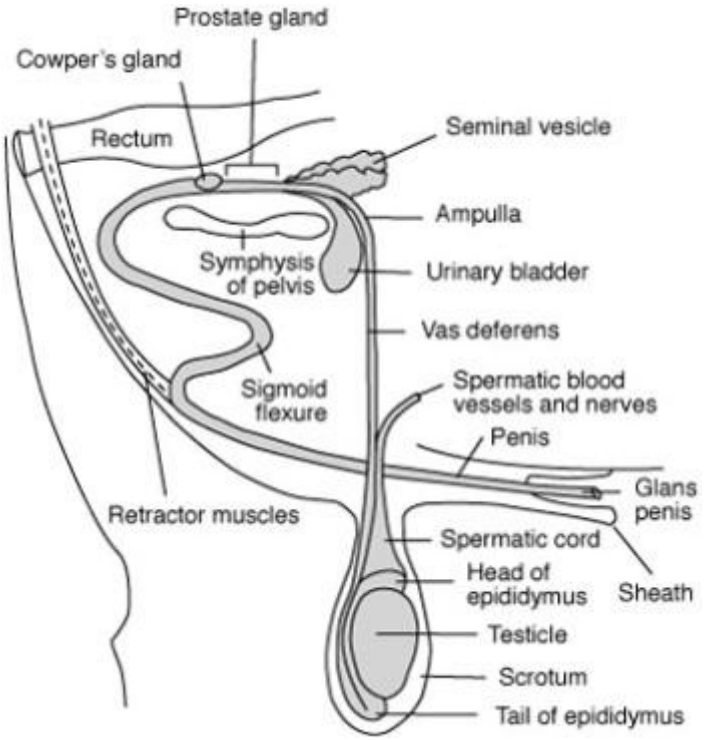

Figure 2.1 The anatomy of a bull's reproductive organs (Articles.extension.org, 2012).

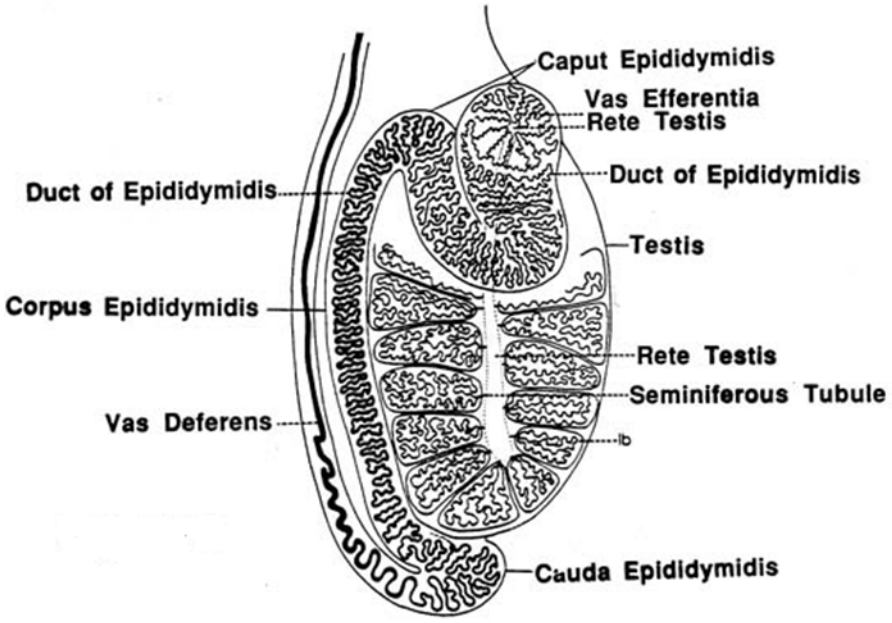

Figure 2.2 Structure of the male testis (Wisconsin-Madison University, 1998).

2.3.1.1 Testes

The testes are located within a sac called the scrotum. The scrotum is vital to the overall functionality of the testes due to its important role in temperature regulation. The external cremaster muscle, located in the spermatic cord regulates how close or far the testes are suspended from the animal's body that allows for thermoregulation in the testes (Whittier, 2016). If a temperature lower than the functioning temperature of the testis is experienced, the cremaster muscles will contract and position the testes closer to the body in order for the temperature of the testes to increase, the opposite occurs when the testes is experiencing a high physical temperature. It is vital for the temperature of the testes to be regulated in order for successful spermatogenesis (i.e. spermacytogenesis and spermiogenesis) to occur and to prevent the degeneration of spermatozoa during high temperatures. The structure of the scrotum that aids in temperature regulation is known as the *tunica dartos* (temperature sensitive layer) (Whittier, 2016). The tunica dartos divides the scrotum into two with each testis being present in one half of the scrotum (Jacobs, 2008). The *pampiniform plexus* is a coil of testicular veins that are responsible for counter-current heat exchange to ensure that the testes do not overheat (Rgd.mcw.edu, not dated. Whittier, 2016).

The main functions of the testes are to produce spermatozoa as well as the male sex hormone, testosterone. Testosterone is produced by the cells of Leydig that are located between the seminiferous tubules. The functions of testosterone are not only limited to sexual reproduction but also plays a role in the regulation of fat distribution in a male animal,

the overall muscle mass of the male, as well as red blood cell production. The testes also contain Sertoli cells that play an essential role in spermatogenesis (Griswold, 1998).

2.3.1.2 Secondary sex organs

The secondary sex organs which include the penis, epididymis and vas deferens all play different roles when it comes to the male reproductive system.

The penis

The penis consists of the urethra which is not only responsible for depositing urine from the bladder to the external environment but is also responsible for the transport of spermatozoa from the *vas deferens* to the female's reproductive tract during natural mating. The penis also contains spongy tissue that is made up of the *corpora cavernosum* and *corpus spongiosum* that become engorged with blood during an erection. The end of the penis is known as the glans penis, which has a rich supply of nerves that are stimulated during mating to result in an erection that is required for ejaculation to occur (Whittier, 2016). Attached to the penis is the sigmoid flexure that is responsible for keeping the penis within the sheath during times that mating does not occur, and extending it out of the sheath during mating. The storage of the penis in the sheath protects the organ against dehydration and infection that might occur if it is exposed to the outer environment (University of Missouri-Columbia, n.d, Whittier, 2016.)

The epididymis

The epididymis is a tubular structure located alongside each testis. The epididymis is convoluted and can be divided into three parts, i.e. the head (*caput*), body (*corpus*) and the tail (*cauda*) (Bertol, 2016; Whittier, 2016). Testicular spermatozoa are only capable of fertilisation once maturation within the epididymis occurs, thus obtaining the potential to fertilise an oocyte (Cosentino & Cockett, 1986; Bertol, 2016). Functions of the epdidiymii, apart from sperm maturation, include transport of spermatozoa from the testis to the vas deferens, absorption of excess testicular fluid in order to increase the overall spermatozoa concentration, the storage of spermatozoa prior to ejaculation, and the degeneration of spermatozoa that are not ejaculated (Whittier, 2016).

The vas deferens

The *vas deferens* is a tubular structure responsible for transporting spermatozoa from the epididymis to the urethra. This tube can also be referred to as the *ductus deferens* and

spermatozoa are passed through this tube via smooth muscle contraction (Missouri-Columbia, n.d; Whittier, 2016; KenHub, 2019).

2.3.1.3 Accessory glands

Semen is a mix of spermatozoa and seminal plasma, with the latter that plays an important role in the nutrition as well as transport of spermatozoa. The glands responsible for the production of seminal plasma include the seminal vesicles, bulbo-urethral glands, prostate gland, and ampulla.

The seminal vesicles

The seminal vesicles secrete the largest proportion of seminal plasma that will aid in the sperm transport from the vas deferens to the urethra during ejaculation. This gland is situated at the end of the vas deferens just before the spermatozoa enter the urethra (Missouri-Columbia, n.d, Whittier, 2016). The seminal vesicle fluid is slightly alkaline, contains various nutritious factors such as fructose, proteins, enzymes and vitamins that play a vital role in sperm survival (McKay & Sharma, 2019).

The bulbo-urethral glands

These glands are also known as the Cowper's glands, and are located on either side of the urethra, beneath the prostate gland. These glands are responsible for secreting a buffer-like liquid responsible for neutralising the pH in the female reproductive tract to allow for optimum sperm survival. The fluid produced by the bulbo-urethral glands also serves the purpose of clearing the urethra of any urine that may still be residing in the urethra prior to ejaculation (Chughtai *et al*., 2005, Missouri-Columbia, n.d, Whittier, 2016).

The prostate gland

This gland is located where the ureter changes over into the urethra. This gland secretes an alkaline solution that acts as a buffer in both the male as well as the female reproductive system. Along with its buffering properties, this gland also secretes a nutrient-rich fluid responsible for nourishing the sperm (Hoffman, 2014, Missouri-Columbia, n.d, Whittier, 2016).

It was previously thought that seminal plasma was necessary for spermatozoa to function successfully (Leal *et al.*, 2018). Vilela *et al*. (2017) however found that in the case of bison (*B. bison*) this is not the case. In their study, bison or cattle seminal plasma were added to bison epididymal spermatozoa, and resulted in no beneficial effect on pre-cryopreservation or post-thaw progressive motility. Some studies found that seminal plasma can even be

detrimental to a spermatozoon's fertilising abilities (Bergeron *et al.,* 2004; Maxwell *et al*., 2006). Factors that can affect the fertilising ability of spermatozoa include amongst others, motility-inhibiting factor as well as decapacitation factor (Bergeron *et al.,* 2004). Presence of seminal plasma can also have a negative impact on the viability of spermatozoa during liquid or cryopreserved storage (Bergeron *et al.,* 2004).

The ampulla

The ampullae connects to either side of the bladder and to the seminal vesicle ducts. The primary role of the ampulla is aiding in spermatozoa transportation from the epididymis to the urethra. The ampullary gland secretes citric acid and fructose to supplement the semen. These components prevent dehydration of the sperm and maintain sperm viability by reducing chemical compounds (citric acid) and providing nutrients (fructose) to the sperm. (Carvalho *et al*., 2014).

Anatomy of a sperm cell

The sperm cell can be divided into three main sections, namely the head, midpiece and tail (Figure 1.3).

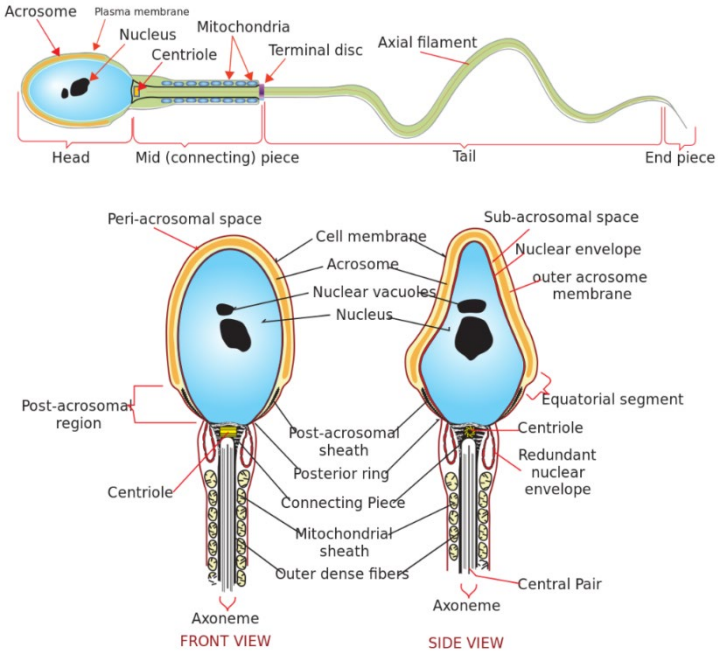

Figure 2.3 The anatomy of a sperm cell (Courses.lumenlearning.com, not dated.).

Head

The head contains the genetic (DNA) material (chromosomes) housed within the nucleus. The head is covered by an acrosome that contains the enzymes hyaluronidase and acrosin that are essential for successful fertilisation. The acrosomal enzymes react with the *zona pellucida* of an oocyte upon sperm contact with this layer. Following this reaction the sperm can then fuse with the oocyte membrane and transfer its genetic material, in the form of a male pronucleus, into the oocyte to ultimately yield a full set of chromosomes after successful fertilisation (Brucker & Lipford, 1995).

Midpiece

The midpiece contains the mitochondria as well as the centriole. The mitochondria provides the sperm cell with the chemical energy needed to fertilise and it obtains ATP (adenosine triphosphate) by converting oxygen and nutrients. Mitochondria play a role in apoptosis, due to it being the site for caspases activation and antiapoptotic and proapoptotic protein interaction which are all required for programmed cell death (Wang & Youle, 2016; Anderson, 2019). The centriole is located between the head and midpiece of the sperm cell (centriole-centrosome complex) and is involved in the aster formation for sperm as well as zygotes. The aster formations are essential for the successful union of the male and female genome (Rawe *et al*., 2008; Anderson, 2019).

The tail

The tail, also referred to as the flagellum, can be divided into 2 main sections namely the principal piece and the end piece. The principal piece contains the axial filament necessary for the motility of the sperm cell. The end piece is responsible for the propulsion of the sperm cell towards the egg. Two main motility patterns that are exhibited by the tail are activated motility and hyperactivated motility. Activated motility consists of the flagella beating gently from side to side, whereas hyperactivated motility consists of more erratic flagellar movement and thus uses more energy (Anderson, 2019).

The flagellum does not immediately hyperactivate once a sperm is ejaculated, with hyperactivation of the flagellum only occuring once the sperm cell is within close range of the ovum. The presence of anandamide near the ovum plays a crucial role in alerting the sperm of the oocyte's presence. Once hyperactivated, spermatozoa will enter a state of capacitation. This state is brought about by the sperm cytoplasm becoming more alkaline, resulting in protons to rapidly move out of the cell due to the pH change (Ickowicz *et al*., 2012).

2.3.2 Spermatogenesis

Spermatogenesis can be defined as the production of mature and functioning spermatozoa by a male animal. Spermatogenesis commences during puberty in the male animal, but it is only when a male animal reaches sexual maturity, when fully functional spermatozoa capable of successfully fertilising an oocyte, wil be produced (Staub & Johnson, 2018).

2.3.2.1 Spermatogenesis process

For spermatogenesis to occur, two essential hormones need to be present, namely testosterone and follicle-stimulating hormone (FSH), the latter which is secreted by the anterior pituitary under influence of gonadotropin-releasing hormone (GnRH). Testosterone has various functions and is produced within the cells of Leydig, which are located in the connective tissue between the seminiferous tubules in the testis (Nazian, 2007; Sherwood, 2013). Testosterone has an effect on the reproductive system prior to birth due to its function in prenatal sex differentiation, however, once the mammal is born testosterone only plays a role again when entering puberty. Puberty is the stage in a mammals life where secondary sexual characteristics start to form, and the reproductive tract starts to mature in order to successfully be able to reproduce (i.e. produce fertile spermatozoa).

Testosterone is produced from a cholesterol precursor molecule and can be classified as a steroid hormone. Testosterone once produced, is transported into the lumen of the seminiferous tubules where it has a vital function in sperm production. The next step, binding of androgen receptors and testosterone, occurs in the cytoplasm of the target cells, from where it gives the command for spermatozoa to be produced (Sherwood, 2013).

Spermatogenesis is a process that produces mature and functional spermatozoa in a male animal, and can be divided into three different stages namely spermatocytogenesis, meiosis and spermiogenesis. Spermatocytogenesis involves the mitosis of spermatogonia thus bringing about the proliferation of these cells, which are then referred to as B-spermatogonia. The B-spermatogonia then undergo further mitosis which leads to the production of primary spermatocytes which enter the second phase known as meiosis (or spermatidogenesis). Primary spermatocytes undergo cell division which results in the production of haploid spermatids. The third stage of this process, spermiogenesis, is considered a maturation stage in which the spermatids undergo nucleus condensation as well as cell stabilisation. During this stage the spermatid cytoplasm is exposed to phagocytosis by the Sertoli cells. Eventually the structures that attach the spermatid to the Sertoli cells ruptures, and this final stage is known as spermiation. Spermiation results in the

spermatozoon entering the tubule lumen of the epididymis (Ownby, 1999; Tapia & Pena, 2014).

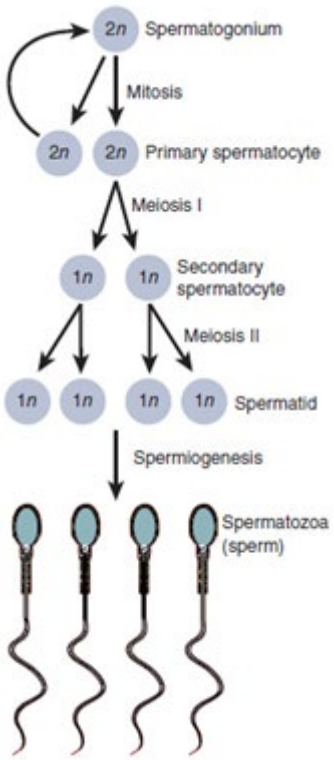

Figure 2.4 The summary of the process of spermatogenesis in male mammal which leads to the production of spermatozoa in male testes (OpenStax college, 2013).

Males of the buffalo species (*Bubalus bubalis*) tend to enter puberty later than domesticated cattle bulls. In *Bubalus bubalis*, the reproductive organs develop prenatally with the organs maturing externally around 6 to 12 months of age (Vale *et al.,* 2014). Water buffalo bulls attain sexual maturity much later than domesticated cattle species (Ahmad *et al.*, 2010; Vale *et al.*, 2014; Selcuk & Akal, 2015). The final achievement of puberty for water buffalo bulls occurs around 24 months of age, compared to domesticated cattle bulls that attain sexual maturity at an age of on average 6-8 months. The duration of the spermatogenesis cycle in buffalo bulls is approximately 75 days, compared to domestic cattle bulls which have a spermatogenesis cycle that lasts approximately 61 days (Vale *et al.,* 2014; Staub & Johnson, 2018).

2.3.3 Maturation of sperm

2.3.3.1 Epididymal maturation

The epididymis is an essential part of the male reproductive system, and can be divided into the *caput* (head), *corpus* (body) and *cauda* (tail) regions (Sherwood, 2013; Cheng *et al*., 2018). Spermatids undergo crucial changes in the epididymus, and should these changes not be induced, spermatids will remain non-functional and thus unable to successfully fertilise oocytes. Several factors involved in the maturation of the spermatids, are synthesized and secreted in the respective regions of the epdidiymus, with the majority of the factors secreted in the *caput* region. The secretions from the epididymis induce the maturation of spermatozoa, which is brought about by the transcriptional silencing of spermatozoa (Bertol, 2016; Cheng *et al*., 2018).

The *caput* and *corpus* epididymal regions are responsible for the maturation of spermatozoa. The *cauda* epididymis has the main function of storing the now matured and functional spermatozoa until ejaculation occurs (Bertol, 2016; Cheng *et al*., 2018). It was often thought that seminal plasma is required in order to mature sperm and allow spermatozoa to be fully functional, however, several studies on mice, humans and cattle indicated that epididymal spermatozoa are fully functional, i.e. are motile and can fertilise oocytes, once it is relased form the cauda epididymus during ejaculation (Mathieu *et al*., 1992; Bongso & Trounson, 1996; Bedford, 2015; Cunha *et al.,* 2019).

2.3.4 Sperm metabolism and energy

The main saccharide found in seminal plasma is fructose, which is an important energy substance utilized by the sperm cell (Tourmente, 2015). In very early studies conducted by Redenz in 1933 (cite Storey, 2008), it was found that in the presence of oxygen the motility of spermatozoa persisted, and that in the absence of seminal plasma, a saccharide such as glucose was required for the conversion to lactate to act as an energy source for the spermatozoa under anaerobic conditions. In some species such as the boar, fructose is the saccharide utilised to convert to lactate during anaerobic conditions, however this resulted in decreased motility when compared to respiration under aerobic conditions with the same saccharide (Storey, 2008).

In later studies it was found that the sperm cells' energy, in the form of adenosine triphosphate (ATP) originates from the mitochondria (i.e. helical structures wrapped around the axoneme) found in the midpiece of a sperm cell (Peterson & Freund, 1970; Storey, 2008; Piomboni *et al*., 2011). The mitochondria is often referred to as the "power house" of the cell

and the biochemical pathways responsible for producing ATP are glycolysis (anaerobic) and oxidative phosphorylation (aerobic) (Peterson & Freund, 1970; Piomboni *et al*., 2011). Oxidative phosphorylation occurs in the mitochondria of the spermatozoa and glycolysis occurs in the fibrous sheath located in the flagellum (tail) of the sperm cell (Peterson & Freund, 1970; Piomboni *et al*., 2011; Tourmente *et al*, 2015). In a study conducted by Peterson & Freund (1970), it was discovered that glycolysis contributes significantly to ATP production. However, some researchers state that it depends on the species as some animals rely on one or the other and some species rely on both metabolic processes (Tourmente *et al*., 2015).

Oxidation during glycolysis is brought about by the supply of carbon atoms to the mitochondria (Piomboni *et al.,* 2011). Glucose molecules are transported through the lipid bilayer of the sperm cell and metabolised into two pyruvate molecules in order to be utilised during glycolysis to yield ATP (Piomboni *et al.,* 2011; Ferramosca & Zara, 2014). Under aerobic conditions, the requirement for a saccharide such as glucose is lowered due to oxidative phosphorylation being the dominant metabolic pathway with regards to production of ATP. However, in the absence of oxygen (i.e. anaerobic conditions) the requirement for glucose increases drastically. Ejaculated sperm converted 18% more glucose to lactate under anaerobic conditions when compared to aerobic conditions. For epididymal sperm, 15% more glucose was converted to lactate under anaerobic conditions when compared to aerobic conditions (Storey, 2008). Buffalo spermatozoa have a poor uptake of oxygen when compared to cattle bulls and thus, under aerobic conditions, a decrease in the overall motility can be seen at a faster rate when compared to the same sample under anaerobic conditions (using glycolytic pathway) (Vale *et al.,* 2014).

2.3.5 Differences between domestic cattle and buffalo bulls

Although domesticated cattle and the different buffalo species (e.g. water buffalo, river buffalo, etc.) are quite similar in terms of anatomy as well as certain behaviour exhibited; important differences in terms of reproductive tract design and function are noted, which in turn indicates that methods developed for cattle cannot simply be adopted for buffalo species. The anatomy of buffalo bulls is similar to that of domesticated cattle bulls, however, the male reproductive organs in the buffalo species tends to be smaller. Buffalo bulls tend to mature later than the domesticated cattle species when sexual maturity is considered. For the process of spermatogenesis to be fully functioning male buffaloes have to be around 24 months of age which is approximately 4 times older than when the domesticated cattle males start undergoing successful spermatogenesis cycles (4-6 months of age), with sperm only being present in the epididymis of male buffaloes at approximately 90 weeks of age

(Vale *et. al.,* 2014). This knowledge is crucial when using ARTs due to the fact that AI and *in vitro* fertilisation protocols require quality viable spermatozoa to ensure the successful use of these ARTs.

2.4 Collection and processing of sperm samples

2.4.1 Harvesting of genetic material from deceased/culled animals

With the conservation of threatened or protected species becoming even more important due to the decline in habitat as well as increase in poaching, it is becoming of utmost importance to investigate different methods in which to obtain the gametes from deceased animals so that their genetic material can be conserved for later use. In male animals, testes are removed from deceased or culled animals to obtain viable spermatozoa. The harvesting of spermatozoa from the cauda epididymis allows for the rescuing of the genetic material of a high genetic merit animal. This conservation of genetic material is especially a valuable method in the case of the loss of endangered/protected animals that are found deceased in the wild, or zoo animals that pass away unexpectedly (Bertol, 2016).

Harvesting or collection of testes on the slaughter line is followed by storage in a closed, cooled container (e.g. a coolerbox with ice packs) at approximately 4ºC until processing occurs, with processing that can be carried out between 0 hours to 72 hours after the passing of the animal. Once the testes are dissected free from the *tunica albuginea,* processing can commence. The spermatozoa are usually harvested from the epididymis due to the sperm that occurs here still being of good quality during normal circumstances as opposed to harvesting from the *vas deferens* (Bertol, 2016). Lambrechts & Hoffman (2018) investigated the effect of cold storage at 4°C for up to 96 hours on Black wildebeest (*Connochaetus gnou*) spermatozoa, with testes removed after the animals were culled. Epididymal spermatozoa were removed from the testes via aspiration and results indicated that spermatozoa could successfully bind to a perivitelline membrane, thus indicating retention of sperm fertilising ability up to 72 hours post-mortem.

2.4.2 Testis collection

According to Hopkins *et al.* (1988), prolonged exposure to degenerating tissue such as that of the testis post-slaughter, could have a deleterious effect on spermatozoa. Due to this reason it was recommended to harvest spermatozoa from the testis as soon as possible. However, since 1988, various methods exploring intact testis cold storage have been investigated. A study conducted by Vilela *et al*. (2017) on bison (*B. b. bison*) indicated that epididymal spermatozoa harvested from bison directly after culling exhibited higher motility

when compared to spermatozoa that were subjected to 24h of cold storage at 5°C when still housed in the epididymis prior to processing and analysis. The spermatozoa that were flushed from the epididymis after 24 h of storage at 5°C exhibited no motility, and were deemed unsuitable for cryopreservation.

2.4.3 Epididymal sperm collection

Mature and fully functioning spermatozoa are stored in the *cauda* epididymis, therefore this region is prefered for the aspiration of epididymal spermatozoa (Cosentino & Cockett, 1986; Bertol, 2016; Whittier, 2016). There are two main methods currently used for the harvesting of epididymal spermatozoa, namely the slicing technique, which is similar to the flotation method, and the flushing technique (Lambrechts *et al*., 1999; Herold *et al*., 2004)

2.4.3.1 Slicing technique

This procedure is a simple and a time-efficient procedure to use. The epididymis is dissected free from the testis and all connective tissue and blood vessels so that the presence of blood does not contaminate the sperm sample. Once the epididymis has been dissected free of all blood vessels and connective tissues, the epididymis is sliced open using a clean scalpel blade. The epididymis can either be left in a diluent post-slicing for the spermatozoa to flow out of the epididymis or multiple slices can be made whilst in the diluent for the spermatozoa to flow out at a faster rate (Lambrechts *et al*., 1999).

2.4.3.2 Flushing technique

In this technique the epididymis and *vas deferens* are dissected free of all connective tissue and blood vessels and a hypodermic needle (usually 23G or 25G) attached to a syringe filled with air is inserted into the vas deferens, and the spermatozoa are pushed/flushed out of the epididymis into a sterile container or tube. This method has been used successfully in an African buffalo study conducted by Herold *et al.* (2004).

2.4.4 Macroscopic evaluation

Macroscopic evaluation refers to the parameters such as the colour, pH and volume of a semen sample that can be evaluated with the naked eye, without making use of a light microscope.

2.4.4.1 Sperm colour

The colour of a freshly collected ejaculate (i.e. spermatozoa including seminal plasma) is usually a milky white to creamy colour, and can even exhibit a slight blue tinge. The general

colour and appearance can vary depending on the individual bull's sexual maturity as well as the season and ejaculation frequency, which all directly affect the concentration of the spermatozoa present in the ejaculate (Vale *et al*., 2014).

2.4.4.2 pH

The ejaculate of buffalo bulls tends to lean towards the acidic range (pH of 6.2-7) due to the composition of the seminal plasma present in ejaculate. Diluents and extenders thus need to have buffering capabilities to prevent fluctuations as well as chemical shock to the spermatozoa during processing, storage and utilisation (Vale *et al.,* 2014).

2.4.4.3 Volume

Ejaculate volume can vary depending on factors that include the degree of sexual maturity of the male, the season (rainy season results in higher ejaculate volumes), ejaculation frequency, and the breed of buffalo (i.e. *Syncerus caffer* or *Bubalus bubalis*). In research conducted by Vale *et al.* (2014), it was found that the normal ejaculate volume for buffaloes was up to 4.5mL, although fully sexually mature bulls could produce up to 8mL of semen in a single ejaculate.

2.4.5 Microscopic evaluation

Microscopic evaluation of sperm samples provide more detailed and specific information on the functional and structural integrity of spermatozoa. Microscopic evaluation also allows for the calculation of the volume of sample required for insemination of *in vitro* fertilisation (Valle *et al*., 2012).

2.4.5.1 Concentration

The concentration of spermatozoa is usually determined using a haemocytometer, with the sperm often diluted first in order to facilitate counting. The dilution rate is then taken into account during the final concentration calculation. Concentration for buffalo bull semen usually ranges from 534 x 10^6 to 1400 x 10^6 spermatozoa per millilitre (Vale *et al.*, 2014).

2.4.5.2 Viability

It is important to determine the viability of a sperm sample due to the viability being directly correlated to the overall quality of the sample, and the potential success of fertilisation. In semen freshly collected for example using the artificial vagina or by using electro-ejaculation, sperm viability can range between 60 to 95%. Sperm samples that have a viability lower than 70% are often not considered suitable for cryopreservation (Vale *et al*., 2014).

2.4.5.3 Sperm motility

Sperm motility can be evaluated in terms of mass motility (looking at cloud movements), individual motility, and progressive motility where the ability to swim forward in a straight line is analysed.

Cloud movements can be analysed subjective by assigning a motility score ranging from 0-5 (i.e. 0 representing samples with no visible motility, and 5 representing strong cloud movements) or objectively by using computer-assisted semen analysis (CASA) software (Palacin *et al*., 2013). Semen samples with a motility of 70% and higher are considered good quality and thus suitable for cryopreservation. In some instances, an ejaculate is static (i.e. no spermatozoa are swimming) but after addition of an extender or diluent motility returns, which thus allows for the evaluation of the sample. Motility, like many of the other sperm characteristics, can be negatively affected by the season, ejaculation frequency as well as sexual maturity status of the bull (Vale *et al*., 2014).

2.4.5.4 Morphology

Morphology refers to the physical structure of spermatozoa, and during analysis the different types of abnormalities in a given sample are recorded. Abnormalities that can be the result of either environmental effects or genetic factors, are classified according to head, midpiece and tail abnormalities. The percentage of abnormalities in a sample can be influenced by season, breed, ejaculation frequency as well as stress experienced prior to ejaculation. It is normal for up to 20% of a sperm sample to consist of abnormal spermatozoa (Vale *et al*., 2014). A high percentage of abnormalities will impact severely on the ability of the sample to be used for AI or IVF, or be considered for cryopreservation.

2.4.5.5 Morphometry

Morphometry refers to the quantitative analysis of the spermatozoa's size and shape. Morphometry can be analysed through computer-assisted methods in order to increase accuracy, which is sometimes lacking when subjective analysis is used (Valle *et al*., 2012; Palacin *et al.*, 2013). To reduce the occurrence of subjective morphological analysis, computer-assisted sperm morphometry analysis systems were developed, with these systems forming part of CASA. The use of CASA involve three procedures namely specimen preparation, image aqcuisition, and image processing and analysis. Morphometric parameters analysed with the use of CASA include the sperm head, sperm nucleus, sperm acrosome, sperm midpiece and sperm flagellum (Yaniz *et al.,* 2015). Due to subpopulations existing within an ejaculate, analysis of homogeneous subpopulations which consists of a

Principal Component Analysis (PCA), should be carried out as well. Sperm subpopulations are classified according to morphometric properties (Valle *et al*., 2012).

2.4.5.6 Acrosome Integrity

Acrosome integrity is one of the most important parameters to analyse due to an abnormal or disintegrated/detached acrosome resulting in the inability for spermatozoa to fertilise the oocyte. In freshly collected water buffalo semen samples, acrosome abnormalities ranged from 5 to 18%, with these samples characterized by lower concentrations of the enzymes acrosin and hyaluronidase that are both required for successful fertilisation of an oocyte (Vale *et al*., 2014).

2.4.6 Flow cytometry and sperm analysis

Flow cytometry is a sensitive and objective method used in the detection of certain pre-determined cell parameters (Jahan-Tigh *et al.,* 2012). This highly repeatable method is most commonly used to quantify parameters of various tissues such as blood, bone marrow samples, bacteria and tissue cultures (Jahan-Tigh *et al*., 2012). Various fluorescent dyes can be used to quantify spermatozoa quality parameters which includes but is not limited to viability, acrosome integrity, DNA content, etc. (Cordelli *et al*., 2005). The fluorescent dyes used during spermatozoa analysis are able to label various parts of a cell depending on the type of analysis required. Components that can be analysed are those such as the antibodies, DNA or the plasma membrane of cells (Jahan-Tigh *et al.,* 2012). Flow cytometry has also been successfully utilised in the sexing of sperm samples, which involves the sorting of the X- and Y-chromosome-bearing spermatozoa (Johnson & Welch, 2000).

During flow cytometric analysis, cells suspended in a solution move at a rate of at least 10 000 cells per second past lasers, thus allowing for the rapid and sensitive quantification of sample populations (ThermoFisher Scientific, not dated.). The scattered light emitted by the lasers is filtered by mirrors for signal amplification once the light has reached the photodetectors. The wavelength of the laser determines which fluorophores on the cell are chosen (Jahan-Tigh *et al*., 2012).

Sperm analysis using flow cytometry is generally conducted using a double stain consisting of a probe that is plasma membrane impermeable or a triple stain that includes the ability to analyse acrosome integrity due to the addition of lecithin (Torres *et al*., 2016). For viability analysis a common dye/probe used is propidium iodide, due to its ability to not permeate membranes. Due to this characteristic, the dye will only enter damaged cells and thus only these cells will emit a red fluorescence (Martinez-Pastor *et al*., 2010). Limitations of flow cytometry include the requirement for cells to be in a single-cell suspension thus eliminating

the posibility for cell-cell interaction analysis. Flow cytometry analysis produces large datasets thus increasing the difficulty of accurate analyses (Jahan-Tigh *et al*., 2012). Flow cytometry has been used for various sperm-related research topics including the analysis of boar sperm (*Sus domesticus*) (Torres *et al*., 2016), Nili-Ravi buffalo (*Bubalus bubalis*) sperm (Li *et al*., 2012), and to determine sperm concentration and plasma membrane integrity of zebrafish (*Danio rerio*) sperm samples (Yang *et al*., 2016).

2.4.7 Short- and long-term storage of spermatozoa

Short-term cold storage of spermatozoa involves storing the spermatozoa in a nutrient-rich medium at a temperature of ±5°C. This storage is done in order to store sperm for further processing or to store sperm for transport prior to use for IVEP or AI. This type of storage also removes the necessity for liquid nitrogen as well as cryo-tanks required for long-term storage, which can not only be difficult to source in some areas (e.g. rural) but can also be rather costly (Raseona *et al*., 2017).

An important consideration during the short-term cold storage of spermatozoa, is the provision of an external energy source for the spermatozoa. Due to the spermatozoa not being immobilised, the sperm cells remain motile and thus a reduction in available energy occurs. Another factor to consider is the fluctuation of the pH that can occur in the diluent that the spermatozoa are stored in, for pH fluctuations can negatively impact on motility and capacitation status, and membrane integrity of sperm. It is speculated that decreased Na^+/K^+-ATPase activity brought on by a decrease in pH could be the reason for lowered motility in acidic environments (Zhou *et al*., 2015).

Long-term storage, also known as cryopreservation, is the process where extremely low temperatures are used in order to freeze or cryopreserve biological samples such as spermatozoa, blood, tissues etc. The most common substance that is used for cryopreservation is liquid nitrogen (LN_2). Important considerations with regards to long-term storage are the formation of intracellular ice crystals as well as cell membrane damage due to exposure to the extremely low temperature of -196°C.

2.4.7.1 Diluents and cryodiluents

Diluents can be used to dilute sperm samples to achieve the desired concentration that will allow for ease of sampling, laboratory experiments, calculations, storage etc. Diluents can either be used for liquid storage or for use in cryopreservation protocols. Diluents are formulated to be rich in nutrients, and often possess a buffering capacity to create the optimum environment for spermatozoa. Nutrient-rich Ham's F10 is an example of a liquid semen extender used for short-term cold storage of spermatozoa (Lambrechts *et al*., 1999).

In a study conducted by Raseona *et al.* (2017), it was concluded that a commercial Triladyl was the best liquid extender for bull semen for short-term cold storage, with Ham's F10 having the second best results. However, the progressive motility observed for semen samples stored using Ham's F10, proved to be higher when compared to samples stored in Triladyl (Raseona *et al.* 2017). Nutrient-rich Ham's-F10 contains sodium bicarbonate that plays an important role in the buffering of solutions in order to maintain a stable pH.

Cryodiluents provide the necessary protection as well as nutrition for the spermatozoa to survive during the process of cryopreservation as well as thawing. There are multiple commercial cryodiluents on the market that contain various ingredients such as egg yolk, skim milk, glycerol etc. Triladyl® is a diluent as well as cryodiluent containing TRIS (a biochemical buffer that is also referred to as Trisaminol), citric acid, sugar, glycerol, water and antibiotics, and requires the addition of egg yolk and distilled water upon mixing the solution prior to use (Minitube, 2018). Triladyl® has proven to be the most successful cryodiluent in studies conducted on African buffalo (Lambrechts *et al*., 1999; Herold *et al*. 2004).

Although egg yolk is an energy-rich ingredient to include in a cryodiluent, there are concerns with regards to the hygiene that could affect the fertilising abilities of the spermatozoa that are stored in such an extender/cryodiluent (Herold et al., 2006). Egg yolk is mainly included in cryodiluents to prevent cold shock from occurring during the cryopreservation/thawing process. The exact mechanisms are unknown, however, it is thought that the low density lipoproteins adhere to the cell membranes of spermatozoa and thus play a role in protecting the spermatozoa against the considerable decrease in temperature during cryopreservation. Another theory is that sperm resilience to cold shock is improved due to the egg yolk preventing the loss of membrane phospholipids. In addition to protecting spermatozoa from cold shock, the egg yolk also increases the motility of spermatozoa post-thaw due to its protective properties (Moussa, *et al*., 2002; Bergeron *et al*., 2004).

The other diluent commonly used is AndroMed® which is an egg-yolk free extender available for the freezing of spermatozoa harvested from ruminants. Andromed® has also been successfully used in the dilution and survival of fresh sperm samples from African buffalo (*Syncerus caffer*). Although concerns regarding potential microbiological contamination are eliminated due to this diluent not containing any ingredients from animal origin, the results achieved for progressive motility, longevity as well as acrosomal integrity were significantly lower than when compared to the same study conducted using Triladyl® (Herold *et al*., 2004). In previous studies Triladyl® has shown to be the most successful diluent when taking viability and overall quality of African buffalo sperm samples into account, when compared to

AndroMed®, Red Ovine Freezing buffer and Sperm-TALP (Lambrechts *et. al*.,1999; Herold *et al*., 2004; Herold *et. al*., 2006).

2.4.7.2 Supplementation

Cryopreserved spermatozoa can suffer membrane damage either due to physical or chemical stress, which decreases the overall quality of the sperm sample and thus reduces the ability of a sperm to participate in fertilisation (Calamera *et al*., 2010; Shah *et al*., 2016). Supplementation of a cryopreservative has the purpose of to try and improve the viability as well as overall quality of the sperm samples post-thaw. Supplements either have nutritive roles or roles that aid in the protection against cryodamage.

Supplementation can refer to the additions of components such as sugars (for example trehalose or glycerol), antioxidants, minerals etc., all with the purpose of ensuring better sperm survival during storage and cryopreservation. Glycerol is a common supplement added to cryodiluents to maintain or even improve sperm quality during cryopreservation. Glycerol is known to decrease the occurrence of physical damage to spermatozoa during the cryopreservation process, however, it has been reported in various studies that glycerol becomes toxic to spermatozoa at higher concentrations leading to lowered fertility and even cell death in extreme cases (Swelum *et al*., 2011; Iqbal *et al.*, 2018). Ethylene glycol is an organic compound that is occasionally used as a supplement during cryopreservation. In a study by Swelum *et al.* (2011) ethylene glycol was used due to its capability of allowing for more rapid onset of cryoprotection when compared to glycerol, thus reducing the osmotic stress that spermatozoa experience during processing for cryopreservation. In their study the overall quality of the spermatozoa sample improved when compared to the fresh samples when compared in terms of viability, acrosomal integrity as well as plasma membrane quality. Sperm motility, however, was not affected positively.

Trehalose is a disaccharide with non-reducing properties. Trehalose not only acts as a nutritive supplement providing the necessary energy supply to the spermatozoa during the cryopreservation process (which includes equilibration, cryopreservation as well as thawing and post-thawing) but also acts as a protective supplement due to it maintaining the osmotic balance of the solution in order to prevent damage to the sperm membrane as well as acting as a membrane stabiliser. Trehalose prevents the formation of ice crystals by dehydrating the spermatozoa, thus preventing the formation of ice crystals that would otherwise cause damage to the spermatozoa and thus lead to poor viability (Shaikh *et al*., 2016; Zhu *et al*., 2017; Iqbal e*t al.,* 2018). Due to decreased ice crystal formation the spermatozoa should exhibit greater viability as well as fertilising abilities when supplemented with trehalose as

opposed to no supplementation at all (Tuncer *et al.,* 2013). In a study conducted on Kankrej bull semen, the addition of trehalose led to improved post-thaw motility, acrosomal integrity as well as plasma membrane integrity (Shaikh *et al.*, 2016). No studies have been conducted on the effect of trehalose supplementation on African buffalo spermatozoa with regards to the viability, morphology and acrosome integrity.

2.4.7.3 Antioxidants

Oxidative stress is an occurrence that can decrease the quality of sperm, with oxidative stress that occurs when the body's antioxidant production is exceeded by the number of reactive oxygen species (ROS) produced. Antioxidants generally present in sperm samples include vitamins C and E, superoxide dismutase, glutathione and thioredoxin, which all participate in the protection of the sperm membrane against the effect of ROS (Ahmadi *et al*., 2016; Park & Yu, 2017).

The sperm plasma membrane is sensitive to damage from ROS because it is rich in poly-unsaturated fatty acids. Spermatozoa exposed to anaerobic conditions experience a potential decrease in quality parameters such as motility and viability (Bucak *et al.*, 2012). Antioxidants neutralise free radicals thus preventing the toxic effect of the ROS on spermatozoa. Methionine, the precursor of glutathione, has been successfully used at various inclusion levels in diluents for ram spermatozoa, and resulted in a protective effect on viability as well as mitochondrial activity up to 96h of liquid storage (Bucak *et al*., 2012). Other antioxidants that have been investigated include green tea extract (Park & Yu, 2017), lycopene (Akalin *et al.,* 2016), and cysteamine (Akalin *et al.,* 2016). Park & Yu (2017) investigated the potential of green tea extract added to cryodiluents to reduce ROS formation during the cryopreservation of porcine sperm. The viability, motility, mitochondrial activity as well as oxidative stress experienced by ram spermatozoa can be improved by supplementing Tris based extenders with 0.5mM and 2mM of lycopene, and 1mM and 2mM of cysteamine during the liquid cold storage of spermatozoa (Akalin *et al.,* 2016).

2.4.7.4 Equilibration period

The time that spermatozoa are exposed to cryodiluents prior to cryopreservation is known as the equilibration period. This period not only allows for the spermatozoa to adapt to the colder temperature over a period of time in order to prevent temperature shock, but the main function is to allow for the spermatozoa to react with the extenders/diluents prior to cryopreservation to allow for the displacement of water to minimise the formation of ice crystals during the cryopreservation (Shah *et al.*, 2016). The duration of the equilibration period is thus vital to the gradual displacement of water from sperm to ensure eventual

successful post-thaw sperm survival. Herold *et al.* (2004) conducted a study that compared equilibration times when cryopreserving epididymal African buffalo spermatozoa using different mediums. The study concluded that an equilibration time between 2 to 9 hours is recommended, as this resulted in the lowest percentage of post-thaw mortalities.

2.4.7.5 Cryopreservation protocol

The method used for cryopreservation is vitally important due to the spermatozoa being extremely sensitive to temperature shock and thus incorrect procedures can lead to a lower quality sample or even a sample that is no longer viable. The most common procedure used for the cryopreservation of animal sperm consists of three important stages.

The first stage includes equilibration which is not only vital in order to prevent chemical shock (as discussed in section 2.1.5) but equally important to gradually reduce the temperature at which the spermatozoa are being stored at. Sperm samples are equilibrated at approximately 4°C for 2 hours in order to gradually decrease the temperature. This is then followed by loading of the French straws (i.e. semen straws), which are subsequently suspended in the liquid nitrogen vapour for 10-15 minutes (Approximtely -80°C) before being plunged into the liquid nitrogen (-196°C) for indefinite storage (Lambrechts *et al.,* 1999; Herold *et al*., 2004; Bertol, 2016; Iqbal *et al*., 2018).

2.4.7.6 Thawing temperature

Thawing temperatures refers to the temperature of the water which the semen/sperm straw is placed in to thaw. The thawing temperature plays an important role in maintaining the cell membrane integrity as well as controlling the osmotic shock that spermatozoa might experience. In a study conducted by Correa *et al*. (1996) various thawing and processing temperatures were considered, and it was concluded that a thawing temperature of 37°C for a minimum time of 30 seconds had the best results with regards to motility. For optimal quality, in their study sperm samples were thawed at 37°C and processed at a temperature of 21°C. Similar results were obtained in a study conducted by Shah *et al*. (2016), in which various thawing temperatures as well as thawing times were investigated. The study concluded that thawing at 37°C for 30 seconds resulted in improved spermatozoa quality than when samples were thawed at 30°C and 75°C for 30 and 9 seconds, respectively.

2.5 Evaluation of post-thaw sperm viability

Post–thaw sperm viability involves the same parameters as that of microscopic evaluation, namely viability, sperm motility, morphology as well as acrosome integrity. The concentration

is not part of the data set, since the concentration was already determined to calculate the dilution rate prior to cryopreservation.

Capacitation is the final maturation step that spermatozoa go through in order to be able to fertilise an oocyte, with this reaction preceded by hyperactivation, and followed by the acrosome reaction. The respective processes all contribute to alterations in the sperm plasma membrane (Cormier *et al*., 1997) as well as the release of the acrosin and hyaluronidase enzymes (Vale *et al.,* 2014). Hyperactivation is achieved due to the increase of tyrosine phosphorylation regulated by a cAMP pathway in the flagellum, which results in the fast, whip-like movements in the sperm tail that assist the head to penetrate the zona pellucida of the oocyte (Talukdar *et al.,* 2017).

Cryopreservation is known to decrease the fertility of spermatozoa due to damage of the plasma membrane occurring as well as altering membrane permeability, which is why capacitation (and thus also the acrosome reaction and hyperactivation) often occurs prematurely (i.e. before reaching the zona pellucida of the oocyte) (Talukdar *et al.,* 2017). It is of vital importance to prevent the onset of capacitation before the spermatozoa is in close proximity of the oocyte in the female reproductive tract (in the case of artificial insemination) or at the site of fertilisation during IVEP.

Premature capacitation leads to the inability for spermatozoa to successfully fertilise the oocyte and death of spermatozoa consequently occurs. Death of spermatozoa occurs due to capacitation affecting the sperm plasma membrane and destabilising the surface of the membrane (Cormier *et al*., 1997; Ickowicz *et al.,* 2012). Once capacitation occurs spermatozoa experience an increased level of membrane permeability, which in turn leads to increased intake of calcium ions thus resulting in the initiation of the acrosome reaction (Cormier *et al*., 1997; Ickowicz *et al.,* 2012). In the case of premature capacitation, this reaction occurs prior to reaching the oocyte and consequently results in decreased fertility. Decreased fertility is the result of the premature relase of acrosin and hyaluronidase, thus rendering the sperm incapable of fertilizing the oocyte (Cormier *et al*., 1997; Ickowicz *et al.,* 2012; Vale *et al.,* 2014). Premature capacitation thus needs to be prevented and can be avoided by the addition of cryoprotectants to the freezing media (Iqbal *et al*., 2016; Shaikh *et al*., 2018).

2.6 Factors affecting sperm quality and viability

Sperm quality can be summarised as the ability for spermatozoa to successfully fertilise an oocyte. This ability would include all parameters influencing the fertility such as viability, morphology, motility, morphology, morphometry and acrosome integrity. The viability is

considered the amount of living sperm cells compared to the dead sperm cells. Viability can be recorded as either the percentage of live sperm cells or dead sperm cells in a sperm sample.

2.6.1 Stress

Stress can be defined as a state in which homeostasis is disrupted or perceived to be disrupted (Tilbrook *et al*., 2000). A large contributing factor to stress of various game/wildlife animals is the capture and translocation of these animals. There are various reasons for needing to translocate animals among which are population control, research, disease and herd health monitoring (such as for bovine tuberculosis), the capture of orphaned or injured animals unable to survive in the wild on their own or the introduction of new species to an area (or re-introduction if the animal has not occurred in that area for a certain amount of time) (Lekolool, 2012). Due to the increasing interest in the capturing and trade of game/wild animals in South Africa over the few decades, more emphasis has been placed on researching capture and handling methods to greatly reduce the stress and inevitably also the mortalities that may occur during these operations (Laubscher *et al*., 2015). Historically, various methods have been used to capture game animals such as using a noose around the neck to immobilise an animal or having holes dug along regularly frequented animal pathways in order for the animal to fall into these holes and thus be captured. Both these methods not only resulted in a high number of injuries to the animals but also posed a great risk to the rangers who were involved in these captures (Lekolool, 2012).

Stress and mortalities not only affect the well-being of the animals but also has a direct effect on the profitability of a game capture operation, thus it is vital to use capture techniques that decrease the possibility for injury or death. Currently the main capture approach being used is a funnel and boma technique in which the animals are herded into a funnel which leads into a closed off area, with this technique the movement of animals can be controlled, sedation of animals can be done in an enclosed and safe area, animal groups stay together and it minimises the contact that the animals have with the handlers (thus reducing the overall stress experienced by the animals as well as rangers) (Lekolool, 2012).

Environmental stress such as climate, nutrition and location has been linked to an overall decrease in sperm quality. The reduction in sperm quality refers specifically to decreased viability of spermatozoa, decrease in motility of sperm, a higher number of cytoplasmic droplets occurring on the midpiece or tail of the spermatozoa as well as increased damage to the sperm acrosome. All these occurrences can ultimately be linked to poor functioning of the epididymis due to chronic stress (Coubrough, 1985; Alejandro *et. al*., 2014).

2.6.2 Pharmaceutical substances

It is common practice to administer various drugs to animals during capture and translocation activities to decrease the risk of injury to rangers and animals alike, as well as to decrease stress levels experienced by the animals. Neuroleptic drugs such as Stresnil (active ingredient azaperone) or M99 (active ingredient oripavine hydrochloride) are administered to achieve sedation or tranquilizing of the animals in question. Administration of drugs such as M5050 (active ingredient diprenorphine) is normally administered to counteract the sedative or tranquilizing drug (Blane *et. al.,* 1967). According to a study conducted on Wistar rats by The World Health Organisation (WHO,1998) the use of azaperone did not have any negative effects on the fertility of adult male rats. When the side effects of Stresnil, M99 or M5050 are considered, no indications of the drug having an effect on the fertility of an animal, is given.

2.6.3 Season and nutrition

The season and climate of an area has a great impact on the reproductive success of an animal species including buffalo herds. This can be due to numerous factors such as the temperature affecting the animal's behaviour, gamete quality, serotonin levels due to duration of photoperiod or the climate affecting quality and availability of grazing.

The semen composition and viability of buffalo bulls is affected by the season, and this will thus have a direct effect on the successful cryopreservation of the spermatozoa (Bhakat *et al*., 2009; Vale *et al*., 2014). The volume of sperm samples collected from buffalo (*Bubalus bubalis*) is larger when collection took place during the rainy season, and cooler temperatures have been found to have a positive effect on the overall freezability of buffalo sperm samples (Vale *et al*., 2014). Buffalo bulls naturally have a much lower libido than domesticated cattle bulls, which will be even less pronounced during periods of extreme temperatures (i.e. heat stress) (Vale *et al*., 2014; Perumal *et al*., 2016).

Thermal stress may result in decreased activity in bulls, with the animals conserving energy for thermoregulatory purposes, which may result in a deminished expression of libido. Other reasons for reduced libido during hot, summer months could be changes in the plasma concentration of steroid hormones and luteinizing hormone which play an integral role in the production of testosterone by the Leydig cells in the testis, as well as decreased functioning of the thyroid (Perumal *et al.,* 2016).

African buffalo cows (*Syncerus caffer*) cycle approximately every 23 days and oestrus lasts between 5 and 6 days. Calving season will be determined by the region's rainfall (Furstenburg, 2010). In buffaloes the breeding season is usually during the wet season so

that calves are born 340 days later during the same season. In the Kruger National Park, breeding occurs from December to April, however in East Africa the breeding season is from February to October (Furstenburg, 2010). Breeding during the wet season ensures that there is sufficient grazing during the initial breeding stage, this allows for the buffalo cow to be in peak condition during ovulation, conception as well as the first few vital months of pregnancy. Another factor is that there is enough grazing for the African buffalo cows to graze on in order to maintain their milk production once parturition has occurred, as well as for the calves to feed on during their first few critical months (Turner *et al*., 2005; Christoph, 2015).

2.6.4 Diseases

2.6.4.1 Foot and mouth disease

Foot-and-mouth disease (FMD) is an infectious disease caused by an *Aphthovirus*. Foot-and-mouth disease can be passed on from African buffalo to domestic cattle through close contact as well as through cryopreserved semen, thus all semen should be tested if to be used to estblish or maintain a disease-free herd. Due to this disease being highly contagious it is preferred not to transport African buffalo to areas of livestock production (Laubscher *&* Hoffman*,* 2012; Jori *et al*., 2016; Perumal *et al*., 2016). A study was conducted in Kenya by Chaters *et al*. (2018) on the effect that FMD had on the fertility of a dairy herd. Within the herd it was concluded that overall fertility was reduced due to the presence of FMD however the exact reasons as to why are not known yet. Chaters *et al*. (2018) speculated that the lowered fertility could be due to increased inflammatory mediators or even due to the presence of pyrexia and lesions in the mouth, resulting in a decreased feed intake and thus having a direct negative effect on the fertility and conception rate.

2.6.4.2 Corridor disease

Corridor disease is caused by *Theileria parva* which is transmitted by infected ticks and it is an indigenous disease that affects cattle as well as buffalo. The ticks become infected by parasitizing on infected African buffalo's and transmit it to domestic cattle upon attachment and feeding. This disease not only poses an economic risk but is also result in fatalities in infected cattle (Laubscher *&* Hoffman*,* 2012; Mitchell, 2018). It is considered one of the most economically important diseases due to it being acutely fatal. Due to this reason not a lot of research has been conducted considering the effects on fertility and breeding due to very few animals surviving the disease once they have been infected (Laubscher & Hoffman, 2012).

2.6.4.3 Bovine tuberculosis

Bovine tuberculosis is caused by the *Mycobacterium spp*. with the most common bacteria that causes infection in African buffalo being *Mycobacterium bovis*. *Mycobacterium bovis* is a zoonotic pathogen which leads to extra emphasis being put on the reduction of occurrences as well as potential eradication of the disease (Michel *et al.* 2006; DAFF, 2016). The disease is spread by infected bodily tissues and milk as well as through contact of infected mucus with water and feed (roughage or grazing). The disease results in various losses such as decreased profits due to having to condemn infected carcasses, unable to sell infected milk, loss of offspring from infected individuals as well as a considerable drop in the reproducing capabilities of the animal. The fertility of infected animals can drop by as much as 11% (often due to genitalia being affected) and in some cases infection can be so severe that the animal becomes sterile. Infected males can also fall victim to orchitis which further reduces the reproducing capabilities of these animals (Michel *et al*., 2006; DAFF, 2016; Perumal *et al*., 2016). The disease has European origins through the importation of European cattle breeds. The disease was first recorded in South Africa for the first time in the late 19th century, the disease saw an increase in occurrence once production systems became increasingly more intensive (i.e. more animals on a smaller land footprint). The African buffalo is considered a maintenance host of *Mycobacterium bovis* which means that the infection remains within the population even without external reinfection occurring (Renwick *et al*., 2007).

2.6.4.4 Brucellosis

Brucellosis causes the female animal to experience an abortion in the last half of gestation resulting in a huge economic loss, thus regulating the disease is becoming increasingly important (Laubscher & Hoffman, 2012; Gorsich *et al*., 2015; Ducrotoy *et al*., 2017). Despite the main problem being contagious abortion this disease impacts bulls as well. Infected bulls exhibit decreased libido, reduction in fertility or complete infertility and epididymoorchitis (the swelling and inflammation of the epididymis) and thus can have a negative effect on the reproductive capabilities of males (Akinci *et al.,* 2006; Perumal *et al*., 2016). Various studies have been conducted on whether brucellosis affected males have decreased sperm quality. A study conducted by Ridler & West (2001) on red deer stags showed that infected males had sperm with significantly decreased forward motility as well as detached heads when compared to sperm of healthy individuals.

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Chapter 3

Methodology

Ethical clearance for the study was obtained from the Animal Ethics Committee of the University of Stellenbosch (Protocol Number: ACU-2017-0477). All procedures and animal handling techniques were carried out in such a manner as to adhere to the guidelines set out by the South African National Standard for the Care and Use of Animals for Scientific Purposed (SANS 10386:2008).

3.1 Experimental location

The collection of samples took place in the Hluhluwe-iMfolozi game reserve during the 2018 Bovine Tuberculosis monitoring operation. The reserve is located in the northern part of the Kwa-Zulu Natal Province, and is characterized by a humid, subtropical climate. This region experiences hot, wet summers and mild, dry winters, with an average annual temperature of 19.5°C and an average annual rainfall of 957mm.

Post-thaw evaluation was carried out in the Animal Physiology laboratory of the Department of Animal Sciences of Stellenbosch University, Stellenbosch, Western Cape. This area is characterized by a Mediterranean climate and experiences cool, wet winters and hot, dry summers. This area is characterised by an average annual temperature of 16.4°C and an annual rainfall of 802mm (Climate-data.org, 2019).

3.2 Experimental material

Testes used in this study were collected from African buffalo (*Syncerus caffer,* N=114) that were culled using a rifle shot and as part of a Bovine Tuberculosis monitoring program (with animals testing positive for bovine tuberculosis being eradicated) in the Hluhluwe-iMfolozi Park, animals culled consisted of both sexes. Testes were collected from 26 adolescent and mature bulls with 21 mature bulls contributing to this study, due to the adolescent males not producing functioning sperm. Three separate herds, identified as Herd A, Herd B and Herd C, which consisted of adults, young adults, sub adults, juveniles and calves of both sexes were culled in the period 17/07/2018 till 02/08/2018. Herds A and B were captured in the same location in the park, and Herd C was captured 8.2km away from this location. Prior to culling, the animals were held in a boma to facilitate sedation for blood sampling and skin tests to determine the bovine tuberculosis status, as well as the extent of infection. Each herd was sedated at different times. During the testing period the animals were exposed to

Stresnil containing azaperone (M99) for sedation, as well as M5050 containing diprenorphine to reverse the sedation. Care was taken to ensure that all animals had *ad lib* access to feed and fresh drinking water during the holding period.

Table 3.1 Summary of the capture and handling dates of the African buffalo herds in the Hluhluwe-iMfolozi Game Reserve during the 2018 Bovine tuberculosis eradication program.

Herd ID	Date Captured	Date 1: ID Tagging, skin tests and blood collection	Date 2: ID Tagging, skin tests and blood collection
Herd A	01/07/2018	02/07/2018	03/07/2018
Herd B	08/07/2018	09/07/2018	10/07/2018
Herd C	14/07/2018	15/07/2018	15/07/2018

Table 3.2 Summary of testis collections, date collected and number of bulls culled during the 2018 trial, testes collected originated from adolescent as well as mature bulls.

Collection Number	Date	Number of bulls
Collection 1	17/07/2018	2
Collection 2	18/07/2018	2
Collection 3	20/07/2018	3
Collection 4	23/07/2018	5
Collection 5	24/07/2018	2
Collection 6	26/07/2018	3
Collection 7	27/07/2018	1
Collection 8	30/07/2018	2
Collection 9	31/07/2018	2
Collection 10	01/08/2018	1
Collection 11	02/08/2018	3

Animals were culled using a rifle shot, and once the animals were culled, they were transported to the abattoir that was approximately one hour from the cull site. On arrival at the abattoir, slaughter commenced, with approximately 8 adult animals that were processed per day. This number however, varied depending on the age and thus physical size of the animal, and how many animals could be accommodated on the slaughter line in the abattoir.

Testes were collected as soon as possible after culling, placed in labelled plastic bags and transported on ice to the laboratory at 4-5°C. The shortest time period from slaughter to arrival at the laboratory was 1 hour and 51 minutes, with the longest transport time being 5 hours and 45 minutes.

3.3 Experimental design

The overall study consisted of four individual experiments, which were structured as follows:

- Experiment 1 investigated the influence of harvesting method and duration of liquid storage on African buffalo sperm viability and quality;
- Experiment 2 investigated the potential of trehalose to minimise deleterious changes that occur during cryopreservation;
- Experiment 3 investigated the effect of thawing temperature and rate on post-thaw African buffalo sperm viability and morphology; and
- Experiment 4 that investigated the potential of flow cytometry to assess post-thaw African buffalo sperm viability.

3.3.1 Experiment 1: The effect of harvesting method and liquid storage on the viability and quality parameters of epididymal African buffalo (*Syncerus caffer*) spermatozoa

The aim of experiment 1 was to investigate the effect of harvesting method and duration of liquid storage on the motility, viability, morphology, as well as acrosome integrity of epididymal African buffalo (*Syncerus caffer*) spermatozoa.

Testes were harvested from culled African buffalo bulls, with the 2 testes from each pair being allocated to two different harvesting methods. One testis was allocated to immediate processing (i.e. 0h intact cold storage) whereas the other testis was allocated to processing after being subjected to 24h of intact storage at 5°C. Epididymal spermatozoa were

harvested from the cauda epididymis using the slicing technique described in section 3.5.1. The testes subjected to 24 h of storage at 5°C were left intact in the tunica albuginea with the epididymis remaining attached to the testis until later processing. The spermatozoa subjected to liquid storage at 5°C were aspirated from the cauda epididymis and diluted to a concentration of 300 million spermatozoa per millilitre using Nutrient-rich Ham's-F10 (Sigma-Aldrich). This was followed by cold storage exposure of 24h at 5°C (Figure 3.1).

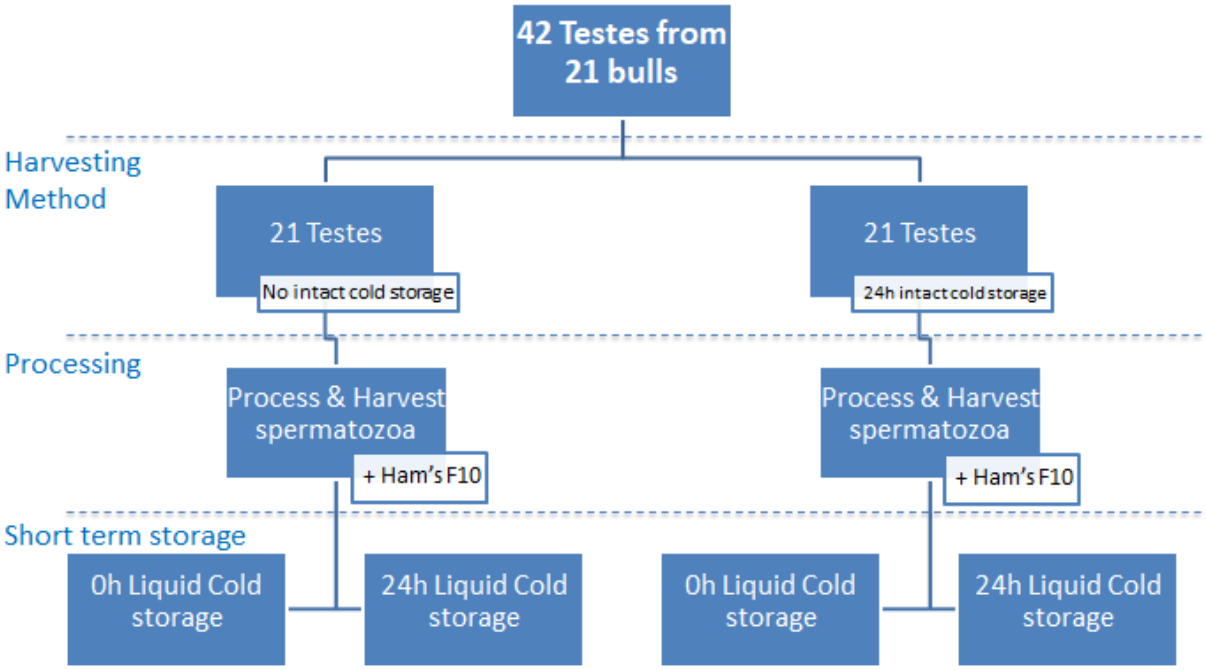

Figure 3.1 The experimental layout for the harvesting method (i.e. cold storage of inact testes) and liquid cold storage (i.e. cold storage of aspirated spermatozoa) of epididymal African buffalo spermatozoa. The testes represented in this diagram all originated from sexually mature bulls.

The experimental design was a 2x2 factorial, and is represented in Table 3.3.

Table 3.3 The experimental design to investigate the influence of harvesting method (i.e. cold storage of intact testes) and duration of liquid cold storage (i.e. cold storage of aspirated spermatozoa) on the viability and quality of epididymal African buffalo spermatozoa.

Harvesting Method	**Duration of liquid cold storage**	
	0 hours	**24 hours**
Testis processed without cold storage (0h)	0h_0h	0h_24h
Testis processed after cold storage (24h)	24h_0h	24h_24h

3.3.2 Experiment 2: The effect of trehalose supplementation on the viability and survivability of epididymal African buffalo (*Syncerus caffer*) spermatozoa

The aim of experiment 2 was to investigate the potential effect of trehalose supplementation on the post-thaw motility, viability, head, midpiece, tail and total morphology as well as acrosome integrity of cryopreserved African buffalo (Syncerus caffer) spermatozoa (Herold et al., 2006; Zhu et al., 2017) (Figure 3.2)

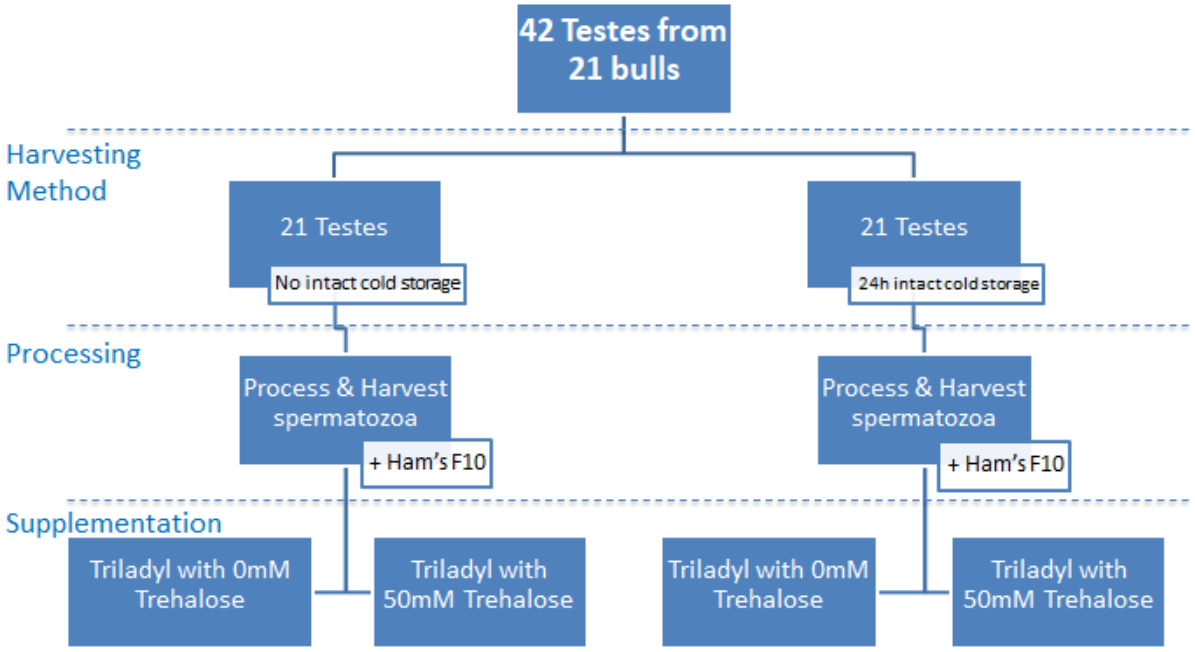

Figure 3.2 The experimental layout for the supplementation of epididymal African buffalo spermatozoa with either 0mM or 50mM of Trehalose pre-cryopreservation. The testes represented in this diagram all originated from sexually mature bulls.

The experimental design was a 2x2 factorial, and is represented in Table 3.4. Epididymal samples were diluted with Nutrient-Rich Ham's-F10, samples that were subject to cryopreservation were further diluted with Triladyl® or Triladyl® supplemented with Trehalose

Table 3.4 The experimental design to investigate the influence of trehalose supplementation to a standard cryodiluent (T+T), namely Triladyl® (Tri), on the viability,morphology and acrosome integrity of epididymal African buffalo spermatozoa. Intact cold storage times are included due to the spermatozoa being used for this experiment originating from testes used in Experiment 1.

Harvesting Method	**Trehalose supplementation**	
	Sperm samples exposed to 0mM of Trehalose	**Sperm samples exposed to 50mM of Trehalose.**
Testis processed without cold storage (0h)	0h_Tri	24h_Tri
Testis processed after cold storage (24h)	0h_T+T	24h_T+T

3.3.3 Experiment 3: The effect of thawing rate on the post-thaw viability and survivability of cryopreserved epididymal African buffalo (*Syncerus caffer*) spermatozoa

The aim of experiment 3 was to determine the influence of two thawing rates, i.e. a fast thawing rate (80°C for 5sec) and the standard thawing rate used for bovine sperm (37°C for 30sec) on the post-thaw motility, viability, and morphology as well as acrosome integrity of epididymal African buffalo spermatozoa (Lambrechts *et al*., 1999) (Figure 3.3).

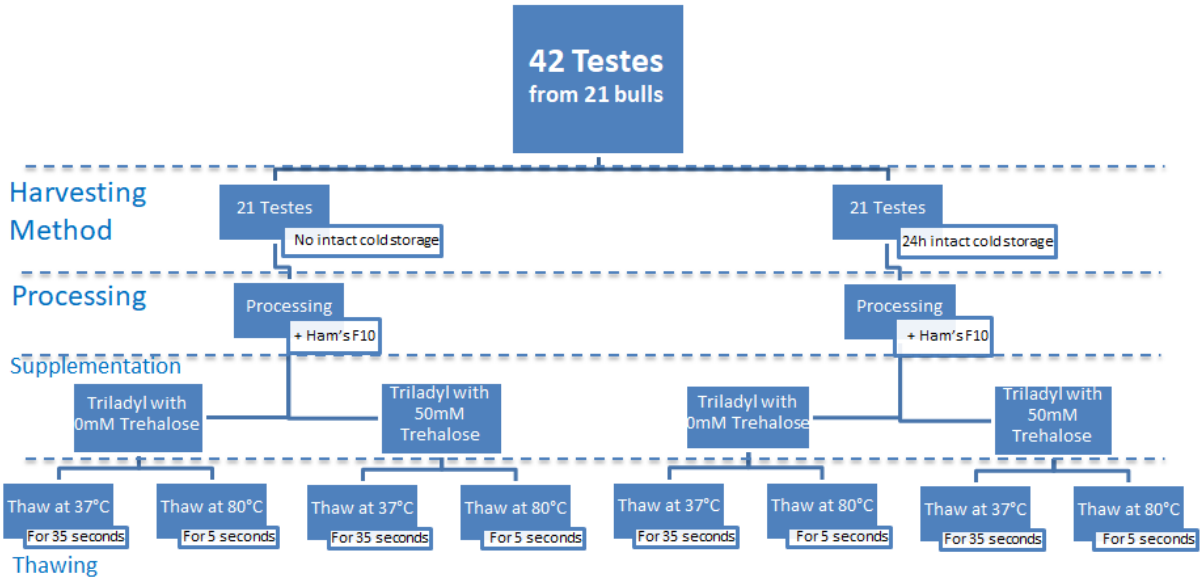

Figure 3.3 The experimental layout for investigating the thawing rate of epididymal African buffalo spermatozoa at either 37°C for 35 seconds or at 80°C for 5 seconds. The testes represented in this diagram all originated from sexually mature bulls.

The experimental design was a 4x2 factorial, and is represented in Table 3.5.

Table 3.5 The experimental design to investigate the potential of trehalose inclusion as well as thawing rate that the cryopreserved samples were subjected to. These treatments are used to discuss both experiment 2 and 3 due to both experiments being represented in the final samples, post thawing.

		Trehalose Supplementation	
Harvesting Method	**Thawing Temperature**	**Sperm samples exposed to 0mM of Trehalose**	**Sperm samples exposed to 50mM of Trehalose**
Testis processed without cold storage (0h)	**37°C**	0h_37_Tri	0h_37_T+T
	80°C	0h_80_Tri	0h_80_T+T
Testis processed after cold storage (24h)	**37°C**	24h_37_Tri	24h_37_T+T
	80°C	24h_80_Tri	24h_80_T+T

The sperm samples were initially aspirated using Nutrient-rich Ham's F10 prior to be being diluted with Triladyl® or Triladyl® supplemented with trehalose. The samples were then cryopreserved and thawed at the two different rates (Table 3.3).

3.3.4 Experiment 4: Quantifying the effect of harvesting method and trehalose supplementation on the viability of African buffalo (*Syncerus caffer*) epididymal spermatozoa using flow cytometry

The aim of Experiment 4 was to quantify the viability of cryopreserved epididymal African buffalo spermatozoa using flow cytometry (BD FACS Melody apparatus) to determine the effect of trehalose supplementation as well as duration of intact storage at 5°C prior to sperm aspiration.

This experimental design was 2x2 factorial, and is represented in Table 3.6.

Table 3.6 The experimental design to investigate the influence of trehalose supplementation to frozen-thawed epididymal African buffalo spermatozoa on viability quantified using flow cytometry.

Harvesting Method	**Trehalose Supplementation**	
	Sperm samples exposed to 0mM of Trehalose	**Sperm samples exposed to 50mM of Trehalose**
Testis processed without cold storage (0h)	0h_Tri	0h_T+T
Testis processed after cold storage (24h)	24h_Tri	24h_T+T

3.4 Preparation of stock solutions and media

3.4.1 Preparation of Trehalose

A stock solution of 100mM trehalose was prepared using trehalose dihydrate powder (Sigma-Aldrich) stored at room temperature, and protected from direct sunlight. To calculate the mass of trehalose dihydrate powder the following formula was used:

$$\boldsymbol{m = C\ x\ V\ x\ M}$$

Where:

m = mass of trehalose dihydrate powder

C = concentration of trehalose (i.e. 100mM)

V = Volume

M = Molecular weight of trehalose (378.33g/mol)

Therefore:

$$m = C\ x\ V\ x\ M$$

$$m = (100x10^{-3}M)x\ 0.200L\ x\frac{378.33g}{mol}$$

$$m = 7.5666g\ of\ trehalose\ dihydrate$$

The required powder sample to prepare 200mL stock solution was weighed using a hypersensitive balance (RADWAG, AS 220/C/2), and then transferred to an autoclaved beaker, whereafter 200mL distilled water was added. The trehalose-water combination was then thoroughly mixed using a magnetic stirrer (Stuart Heat-stir, SB162) until all the trehalose dehydrate was dissolved. The trehalose stock solution was decanted into clearly marked 25mL Falcon tubes and placed in a freezer (-18°C) for storage until the trial took place. Each day and prior to testis processing, an adequate volume of trehalose stock solution was thawed in a water bath (Memmert) maintained at 37°C to prevent temperature shock to the spermatozoa.

3.4.2 Preparation of Triladyl®

Triladyl® (MiniTube) stock solution was used to prepare the working cryodiluent. The work surface was cleaned with 70% alcohol to ensure a sterile working environment. Egg yolk was separated from the albumin, and each egg yolk was then placed on coffee filters in order for any excess albumin to be absorbed by the coffee filter. A sterile disposable syringe (50mL; Lasec, South Africa) was used to extract the egg yolk which was then transferred to a 1000mL sterile beaker. This process was repeated until the beaker contained 200mL of egg yolk.

Deionised water (600mL) was slowly added to the 200mL egg that was continuously stirred using a magnetic stirrer (Stuart Heat-stir, SB162). Care was taken to ensure that no foam formed during the addition of the water. Once all the egg yolk had dissolved and a homogeneous mixture was achieved, 200mL of Triladyl® stock solution was added to the mixture, care was again taken to prevent the formation of foam. The ratio for the Triladyl® diluent was 1 part egg yolk: 3 part deionised water:1 part Triladyl® stock solution.

Once a homogeneous mixture was achieved the prepared Triladyl® mixture was transferred to 50mL Falcon tubes (filled to 45mL to allow for expansion during freezing) and clearly marked with solution name, date and ID of individual who prepared the solution. The tubes were then stored at -18°C until commencement of the trial.

3.5 Processing of samples

3.5.1 Collection and transportation of samples

Prior to testis collection, the respective diluents (Triladyl® and trehalose) were placed in a 37°C water bath to thaw and to reach optimal processing temperature to prevent thermal shock to the spermatozoa during sample processing.

Slaughter start time was recorded in order to monitor the extent of time that the testis was exposed to environmental factors prior to processing. The identification and tuberculosis status of each animal was recorded as the carcass was suspended on the abattoir slaughter line. The reproductive systems of only the adult bulls were collected and the testes were dissected free from the scrotum. The testes were placed into clearly marked collection bags and then stored in a cooler box on ice packs. Access to the cooler box was monitored to prevent temperature fluctuations within the cooler box which could negatively affect the testes. As soon as testis collection was completed, the material was transported to the laboratory for further analysis.

Each pair of testes was then allocated to the two treatments that involved harvesting method and duration of liquid storage (Experiment 1). One testis was processed immediately post-slaughter whereas the remaining testis was subjected to intact storage at 5°C for 24h before being processed. Testes subjected to immediate processing remained in the cooler box pre-processing whereas testes subjected to 24 hours of intact cold storage at 5°C prior to processing were placed into clearly identifiable collection bags and placed in a refrigerator.

For both harvesting methods, the same processing protocol was followed. The testis was dissected free of the *tunica albuginea* and the width, length and circumference of the testis was recorded. The cauda epididymis including a small section of the vas deferens was dissected free from the testis and placed in a clean 90mm Petri dish, and covered in order to prevent dehydration of the epididymis. Two testicular tissue samples were taken from the testis. The tissue was excised by making a cross section through the widest region of the testis, a slice of approximately 15mm wide was removed and two triangular tissue samples were cut from the cross section slice using a no. 23, unused and clean scalpel blade. The samples were then transferred to a specimen vial; whereafter 10% buffered formalin solution was added to preserve the tissue for later histological evaluation.

The length as well as width of the cauda epididymis was recorded before further processing commenced. All connective tissue and blood vessels were removed from the cauda epididymis surface using a no. 11, clean and unused scalpel blade as well as a pincette. As large a portion of the epididymal tubules was then removed, and care was taken to not include the tubules closest to the vas deferens as this could contain dead spermatozoa which could influence the results (Lambrechts *et al*., 1999; Herold *et al*., 2006). The portion of cauda epididymis was placed in a 65mm Petri dish containing 3mL of pre-warmed Ham's F-10 nutrient-rich fluid (37°C). The epididymal spermatozoa were aspirated using a slicing technique, which entailed slicing across the width of the exposed epididymal tubules (Bertol, 2016). Once slicing was completed the cauda epididymis was rinsed with another 1mL Ham's F-10 nutrient-rich fluid, and the epididymal tissue was discarded following a strict biosecurity protocol. The 65mm petri dish containing the epididymal spermatozoa and Ham's F-10 nutrient mixture was placed on a warming plate (Electrothermal, Lasec) set at 37°C in order to prevent temperature fluctuations.

3.5.2 Concentration determination

Samples were taken from the epididymal spermatozoa and Ham's F-10 nutrient-rich mixture to determine the sperm concentration. An aliquot of 10µL epididymal spermatozoa and Ham's F-10 nutrient-rich mixture and 990µL H_2O were added together into a 1mL Eppendorf tube to make a 100x dilution for concentration determination, the tube was everted 2-3 times

to ensure that the sample was adequately mixed. Of this mixture, 10µL was placed on a McMaster slide and the concentration was assessed under a light microscope. Epididymal spermatozoa in 5 of the 25 counting blocks were counted and the result was used to calculate the overall epididymal spermatozoa concentration of the sample (Lambrechts *et al*., 1999).

$$Concentration\ per\ ml = Spematozoa\ counted\ x\ 5\ x\ dilution\ factor\ x\ 10\ 000$$

3.5.3 Making of smears

Nigrosine-Eosin staining material (Kyron Laboratories, South Africa) was used to make duplicate smears of the fresh and frozen-thawed epididymal spermatozoa samples for evaluation of sperm viability, morphology, and acrosome integrity. An aliquot of 50µL epididymal spermatozoa and diluent/cryodiluent mixture was pipetted on to a clean microscope slide and 50µL Nigrosine-Eosin dye was added to the sample, mixed and allowed to react for 30 seconds prior to making the smears. Slides were marked with the date, treatment and animal ID. Smears were allowed to air-dry and then stored for later analysis.

3.5.4 Cryopreservation

3.5.5.1 Dilution and equilibration

The number of straws to be cryopreserved was determined by the sperm concentration of the sample, and the volume of sample available for cryopreservation. A certain volume of sample was also allocated to an IVEP trial that was carried out at the same time. The epididymal spermatozoa and Ham's F-10 nutrient-rich mixture was subjected to two different treatments prior to cryopreservation, namely dilution to a concentration of 30 million epididymal spermatozoa per millilitre using Triladyl® (Lambrechts *et al*., 1999; Herold *et al*., 2006) or dilution to a concentration of 30 million epididymal spermatozoa per millilitre using Triladyl® containing 50mM of Trehalose (stock solution of Trehalose was 100mM).

1. $n\ of\ straws\ desired\ x\ 0.25ml = total\ v\ needed\ (in\ ml)$

2. $\frac{desired\ spermatozoa\ concentration}{actual\ spermatozoa\ concentration} =$
   $v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v$

3. $v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v\ x\ total\ ml\ needed =$
   $total\ v\ of\ spermatozoa\ mixture\ needed$

4. $Total\ v\ needed - total\ v\ of\ spermatozoa\ mixture\ needed = v\ of\ cryodiluent\ needed$

4.1 If cryodiluent used only consists of Triladyl® the total v of cryodiluent needed will be made up of Triladyl®.

4.2 If cryodiluent used consists of a mixture of Triladyl® as well as Trehalose solution, 66.67% of cryodiluent mixture must consist of Triladyl® and 33.33% of mixture must contain 100mM Trehalose stock solution (resulting in Triladyl® with 50mM Trehalose).

The epididymal spermatozoa and Ham's F-10 nutrient-rich mixture was diluted with the selected pre-warmed cryodiluent, maintained in a water bath set at 37°C, by gradually pipetting the diluent into the dilution tube (15mL Falcon tube, Lasec) to prevent potential thermal and chemical shock. The diluted cryodiluent-sperm mixture was gently everted 2-3 times to ensure proper mixing of the sample, before being placed in a 250ml glass beaker containing H_2O, and then equilibrated at 5°C for approximately 3 to 4 hours (Lambrechts *et al*., 1999; Herold *et al*., 2006).

3.5.5.2 French straw loading and cryopreservation

French straws with a volume of 0.25cc were used for the loading of the sperm samples. The straws were clearly marked (information included animal ID, date, sample treatment) and placed in the refrigerator at 5°C in order to reduce the risk of temperature shock to the spermatozoa once the samples were loaded into the French straws after equilibration. Once loaded, each French straw was sealed using PVC powder (Taurus-Evolution, South Africa) After ensuring that the French straws were properly sealed, straws were suspended in liquid nitrogen vapour (-80°C) for 15 minutes followed by submerging into the liquid nitrogen (-196°C) for 5 minutes (Herold *et al*., 2006; Swelum *et al*., 2011). Cryopreserved straws were transferred to clearly marked goblets that were assigned to specific canisters inside the liquid nitrogen tank, and location recorded.

3.5.6 Thawing

On the 12th of August 2018 all the samples and smears were transported back to Stellenbosch University for further laboratory analysis. The cryopreserved sperm straws containing the epididymal African buffalo spermatozoa samples were thawed using two different treatments. The first treatment consisted of thawing the epididymal spermatozoa straws for 35 seconds in a water bath maintained at 37°C, which served as control (Swelum *et al*., 2011) after which the sample was transferred into a 1mL Eppendorf tube. The same

procedure was followed with the second treatment, however, the cryopreserved sperm straws were thawed at a fast thawing rate, i.e. 80°C for 5 seconds (Lambrechts *et al.,* 1999).

3.5.7 Flow cytometry

The dye for flow cytometry analysis were prepared by mixing the SYTO 9 nucleic acid stain and propidium iodide (Molecular probes; L34856) at a 1:1 ratio, and centrifuged to allow for proper mixing (200rpm, 3 seconds) Cryopreserved African buffalo samples with a volume of 0.25cc were thawed at 37°C for 30 seconds, and then transferred to a 2000µL Eppendorf tube. An aliquot of 300µL thawed sperm sample was transferred using a pipette to a clearly marked (ID and Treatment) Falcon tube compatible with the BD FACSMelody (Becton Dickinson, San Jose, USA) machine. An aliquot of 0.6µL of dye mixture was added to the epididymal sperm samples using a pipette, and was gently everted 2-3 times and allowed to stain for 10 minutes. Each tube was vortexed for 1-2 seconds before being placed in the BD FACSMelody machine for analysis. Analysis of the samples was performed by the machine compatible computer program, BD FACSChorus. General analysis included the creation of scattergrams based on the analysed samples. Samples that fluoresced green indicated spermatozoa with intact cell membranes and thus classified as live spermatozoa whereas spermatozoa with damaged membrane, thus dead spermatozoa, fluoresced far less and could even show a red fluorescence. A population referred to as intermediate was also recorded which included spermatozoa that were in-between being classed as live and dead.

3.5.8 Data recorded

All the Nigrosine-Eosin sample slides were analysed for the overall viability (percentage of live epididymal spermatozoa after being exposed to each treatment), morphology analysis as well as acrosome integrity analysis. This was performed using a light microscope as well as an Olympus IX70 microscope (Wirsam, South Africa) with an OLYMPUS cellSens standard camera (Wirsam, South Africa).

3.5.8.1 Testis and cauda epididymis measurements

Measurements of the length, width and circumference were recorded of the testis and cauda epididymis. Values were recorded for future reference if needed.

3.5.8.2 Mass and individual motility

Motility was recorded for fresh, equilibrated as well as post-thaw samples. Each sample was placed on a pre-warmed microscope slide (37°C) and placed on a warming plate maintained at 37°C for 2 minutes to allow for the sample to stabilise (i.e. not exhibit drifting) before

motility was recorded. Each sample was analysed for overall cloud movement, and once the mass motility score was recorded, a cover slip was placed on the sample to prevent drying out and to obtain a clear image of individual motility within the sample. The motility was scored subjectively using a scoring system of 1-5, with 1 referring to little or no movement and 5 referring to good progressive motility and a high percentage of motile spermatozoa in the sample (Herold *et al*., 2006).

3.5.8.3 Viability

The Nigrosine-Eosin smears prepared for the fresh and post-thaw samples were analysed for the viability of the sample. Viability refers to the percentage of live spermatozoa present per slide. At least 100 spermatozoa had to be present per slide to calculate the viability of a smear. Viable or living spermatozoa appeared white whereas non-viable or dead spermatozoa ranged from pink to purple in colour (Figure 3.4).

The percentage of viable spermatozoa was calculated as follows:

$$Live\ Cells = \frac{Total\ live\ cells\ counted}{Total\ cells\ counted} \times 100$$

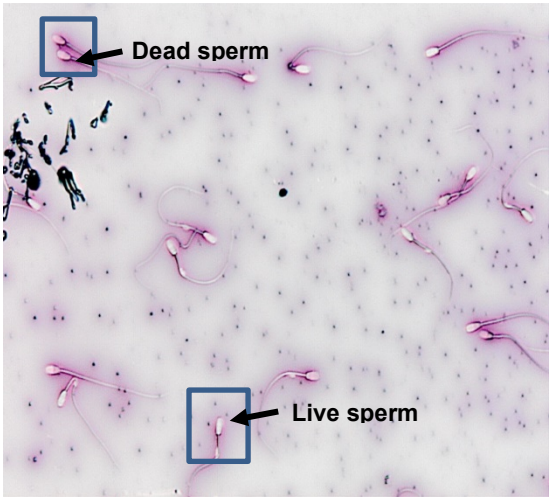

Figure 3.4 A nigrosine-eosine smear indicating the difference between live (white, unstained) and dead (stained pink-purple) epididymal African buffalo spermatozoa.

3.5.8.4 Morphology

The Nigrosine-Eosin smears prepared for the fresh and post-thaw samples were used to analyse the morphology of each sample. Morphology refers to the physical appearance of the sperm cell. The morphologies that were analysed included head, midpiece and tail, and the three classes were pooled to provide the percentage of total percentage abnormal morphologies. A minimum of 100 spermatozoa were analysed per smear, and the respective class of abnormality was calculated as follows:

$$\%\ Abnormal\ morphology = \frac{Abnormal\ morphology\ counted}{Total\ cells\ counted} \times 100$$

Abnormal morphologies that were recorded included (but were not limited to) double heads, detached heads, double tails, coiled tails, bent tails, double midpiece, bent midpiece, cytoplasmic droplets amongst others (Lambrechts *et al*., 1999).

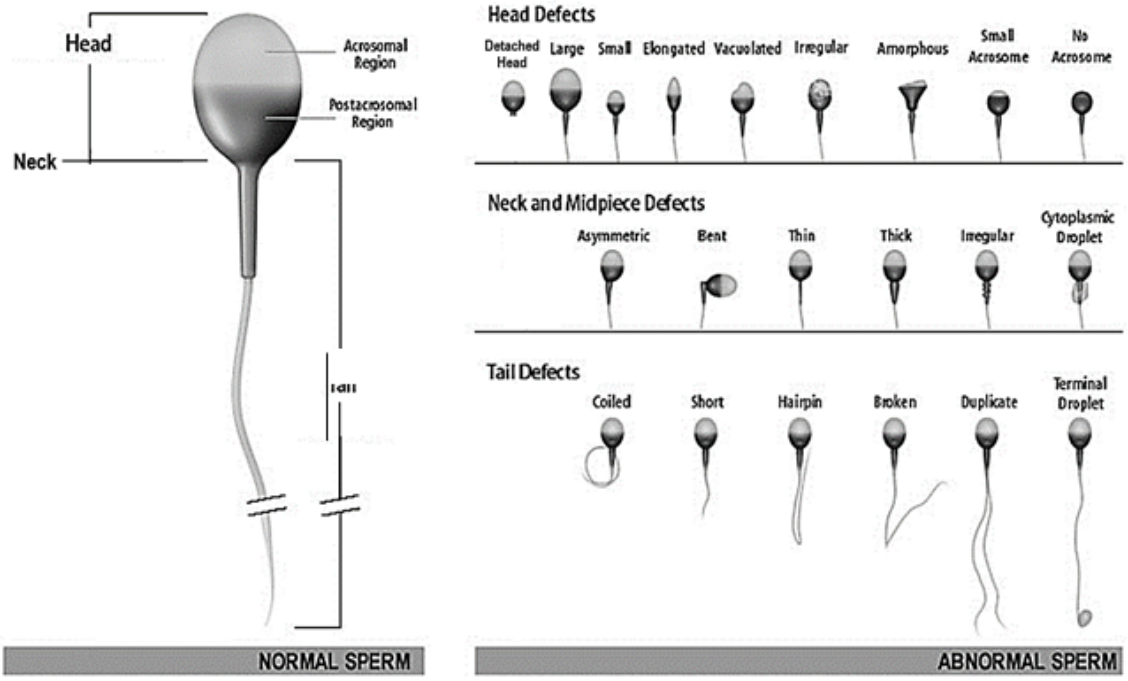

Figure 3.5 Different types of head, midpiece and tail morphological abnormalities that were used to evaluate epididymal African buffalo sperm samples (Source: https://www.carolinaconceptions.com/understanding-sperm-morphology/; 2019).

3.5.8.5 Acrosome integrity

The nigrosine-eosin smears made for the fresh and post-thaw samples were used to evaluate the acrosome integrity of each sample. A minimum of 100 spermatozoa were evaluated per slide to calculate the acrosome integrity, using the following equation:

$$\%\ intact\ acrosomes = \frac{Total\ intact\ acrosomes}{Total\ cells\ counted} \times 100$$

Acrosomes that were considered as being intact were characterized by a clear definite line through the middle section of the sperm head, with the acrosome also defined by a uniform colouring (Herold *et al.,* 2006) (Figure 3.6). Acrosomes that were considered not intact was characterized by colour change within the head of the sperm, textured sperm head, no clear line, fuzzy outer edge to acrosome.

Figure 3.6 Spermatozoa with an intact acrosome, characterized by the define line through the head of the spermatozoa, stained using Nigrosine-Eosin dye.

Figure 3.7 Spermatozoa with a damaged acrosome, characterized by no clear line through head of spermatozoa and no consistent head colouration, stained using nigrosine-eosin dye.

3.6 Statistical analysis

The statistical analysis for this project was performed using XLStat. The data was divided into the fresh samples (i.e. no cryopreservation), cryopreserved samples (cryopreserved in cryodiluents or cryodiluents with supplement) as well as the combined samples (where all the cryopreserved samples as well as the immediately analysed post-processing fresh samples were compared). The non-transformed, log transformed as well as square root transformed data were compared, and the best model was chosen based on the normality, homoscedasticity as well as the coefficient of determination. If the model chosen was transformed the data was transformed back to original format prior to interpretation. All parameters were analysed using descriptive statistics in order to identify and familiarise with the general trend of the data. One-way ANOVA's were used for the analysis of the viability, morphology and acrosome integrity, this analysis included a Bonferroni analysis. The motility was analysed using descriptive statistics due to results not being evenly represented. The difference and potential significance of the difference between the fresh and cryopreserved samples was analysed by using a two-sample paired t-test. A 5% significance level was assumed for all analyses.

3.7 References

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Chapter 4

Influence of harvesting method on African buffalo *(Syncerus caffer)* spermatozoa viability and survivability

4.1 Abstract

This study investigated the influence of harvesting method on the viability and survivability of epididymal African buffalo (*Syncerus caffer*) spermatozoa. Testes were either processed immediately after culling or after a 24-hour storage period. The harvested spermatozoa were subjected to the same treatment protocols, i.e. evaluated immediately after aspiration, and after 24h being maintained in Ham's-F10 storage medium at 5°C. Parameters evaluated included mass motility, viability, morphology and acrosome integrity. The viability of samples ranged from 42.92% to 94.97%, with viability significantly higher in sperm samples not subjected to liquid storage (5°C) post-aspiration. The midpiece abnormalities recorded ranged from 0.00% to 73.00%, with the lowest abnormal midpiece morphology recorded for spermatozoa harvested from testes stored intact for 24h (5°C), and analysed immediately post-aspiration. The tail abnormalities ranged from 0% to 40.59%, and spermatozoa aspirated and evaluated directly after slaughter, demonstrated significantly less tail abnormalities when compared to spermatozoa harvested from testes stored intact for 24h at 5°C. The percentage of total abnormalities recorded ranged from 6.00% to 78.00%, and sperm samples aspirated from testes stored intact for 24h at 5°C were characterized by significantly less abnormalities, when compared to sperm samples subjected to 24h of liquid cold storage (5°C) post-aspiration. The percentage of head abnormalities and acrosome integrity investigated during this experiment were not significantly affected by the treatments used. Significant differences for the survivability, midpiece abnormalities and tail abnormalities were recorded for the herds however; the differences were not isolated to a particular herd. Findings from this study will assist in the refinement of post-mortem harvesting and processing protocols or the long term conservation of germplasm from African buffalo bulls, and also for potential use in assisted reproductive techniques to restore populations.

Keywords: African buffalo, spermatozoa, epididymal, harvesting, aspiration

4.2 Introduction

The African buffalo (*Syncerus caffer*) is a member of the so-called Big 5 and is highly sought after for both eco-tourism as well as trophy hunting operations. In 2015, 12% of the total hunting activity earnings, which equates to R145 million to South Africa's annual foreign exchange currency, was earned due to the hunting of African buffaloes (Coleman, 2018). The African buffalo as a bulk grazer plays an important role in ecosystems due to its utilisation of the long, fibrous grasses thus converting the grazing to short grasslands which is more suitable for smaller herbivores such as the impala (*Aepyceros melampus*) (Michel & Bengis, 2012, Krugerpark.co.za, 2017).

The African buffalo is susceptible to four economically significant diseases namely foot-and-mouth disease, Corridor disease, Bovine tuberculosis (TB) and Brucellosis (Laubscher & Hoffman, 2012). The abovementioned diseases have negatively impacted the production and reproduction of buffalo populations in the Kruger National Park in Mpumulanga as well as Hluhluwe-iMfolozi Game Reserve in Kwa-Zulu Natal (de Vos & van Niekerk, 1969; Laubscher & Hoffman, 2012). It is this imperative that existing protocols for the collection and processing of germplasm obtained from African buffalo, i.e. spermatozoa and oocytes, are refined to ensure the optimal processing and long-term preservation of the genetic material in genome resource banks.

In a study conducted by Hopkins *et al*. (1988), deleterious effects can be present if the spermatozoa remain in the testis for longer periods of time due to the negative impact of the degenerating tissue. Since then various other studies have been conducted on bovines and bison (*Bubalus bubalis bison*). One such a study conducted on bison considered processing testes immediately after harvesting or storing the intact epididymis at 5°C for 24 hours, this study concluded that immediate harvesting and processing lead to the most successful results when compared to cold storage prior to processing (Vilela *et al*. 2017).

The aim of this study was to determine the influence of harvesting method and duration of liquid cold storage (i.e. at 5°C) on the mass motility, viability, morphology and acrosome integrity of African buffalo spermatozoa obtained from testes collected from bulls culled as part of a TB monitoring program.

4.3 Methodology

4.3.1 Experimental Location

The collection of samples took place in the Hluhluwe-iMfolozi Game Reserve during the 2018 Bovine Tuberculosis monitoring operation. The reserve is located in the northern part of the Kwa-Zulu Natal Province, and is characterized by a humid, subtropical climate. This region experiences hot, wet summers and mild, dry winters, with an average annual temperature of 19.5°C and an average annual rainfall of 957mm (Climate-data.org, 2019).

4.3.2 Experimental animals and collection of testes

Testes were collected from African buffalo (*Syncerus caffer*, N=114) that were culled using a rifle shot as part of a Bovine Tuberculosis monitoring program (with animals testing positive for bovine tuberculosis being eradicated) carried out in the Hluhluwe-iMfolozi Game Reserve, animals culled consisted of both sexes. Testes were collected from 26 adolescent and mature bulls with 21 mature bulls contributing to this study, due to the adolescent males not producing functioning sperm. Three individual herds (i.e. Herd A, Herd B and Herd C) that consisted of adults, young adults, sub-adults, juveniles and calves of both sexes, were culled in the period 17/07/2018 till 02/08/2018. Herds A and B were captured in the same location of the Reserve, and Herd C was captured 8.2km away from this location. Prior to culling the animals were maintained in a boma to facilitate sedation for blood sampling and skin tests to determine the bovine tuberculosis status as well as the extent of infection.

The pharmaceutical products used to achieve sedation included Stresnil (azaperone-M99; Sigma-Aldrich) for sedation as well as M5050 (diprenorphine; Sigma-Aldrich) to reverse the sedation. Care was taken to ensure that all animals had *ad libitum* access to feed and fresh drinking water during the holding period. Herds A, B and C were maintained in the boma between 16 to 25 days, 15 to 22 days, and 10 to 19 days, respectively.

4.3.3 Experimental design

The experiment was a 2x2 factorial design, with the respective treatments including aspiration of spermatozoa at 0h and 24h and sample storage of 0h and 24h. The treatments were labelled using the first hour label representing the time at which testes were processed (i.e. 0h post-slaughter or 24h post-slaughter) and the second hour label representing the time the spermatozoa were subjected to liquid cold storage (i.e. 0h cold storage or 24h cold storage). Cold storage throughout the study was performed at 5°C.

4.3.4 Processing of testes

For allocation to the respective treatment groups, the two testes of each bull were collected, and from each set, one testis was processed immediately after culling, whereas the other testis was stored intact and protected against dehydration, at 5°C for 24h before being processed.

Processing of each testes and aspirated sperm samples were carried out at room temperature (about 22°C).The *tunica albuginea* of each testis was cut open to expose the testis and epididymis. After removal of the *tunica albuginea,* testis length, width and circumference were recorded. The cauda epididymis including a part of the *vas deferens* was then detached from the testis, and transferred to an unused 90mm Petri dish, and care was taken to prevent dehydration of the epididymis.

To obtain the epididymal sperm samples, the tubules of each cauda epididymis were exposed by removing the connective tissue and blood vessels using a scalpel blade (size no. 11, Lasec) and a pincette. After exposure, a large portion of the tubules before transition into the vas deferens was removed and transferred to a 65mm Petri dish containing 3mL Ham's-F10 (Sigma-Aldrich) pre-warmed to 37°C (Lambrechts *et al*., 1999; Herold *et al*., 2006). A slicing method was used to allow for the release of the spermatozoa into the collection medium (Bertol, 2016). Once slicing was completed the tubules were rinsed with another 1mL Ham's F-10 (pre-warmed to 37°C), to ensure optimal sperm recovery. The Petri dish containing the epididymal spermatozoa and Ham's F-10 nutrient combination was then placed on a warming plate (MH6616, Electrothermal, Lasec) set at 37°C in order to prevent temperature fluctuations.

The fresh epididymal spermatozoa and Ham's F-10 Nutrient-rich mixture (Lambrechts *et al.,* 1999) was diluted to a concentration of 300 million spermatozoa per millilitre, the desired concentration was achieved by diluting the sample with Ham's F-10 Nutrient-rich mixture after performing a concentration analysis.

Samples of the diluted epididymal spermatozoa and Ham's F-10 mixture from both the 0h and 24h intact testis storage were pipetted into an Eppendorf tube and refrigerated at 5°C for 24 hours in order to assess the liquid storage capabilities of fresh epididymal spermatozoa samples.

4.3.5 Data recorded

Testis and cauda epididymis measurements: Measurements of the length, width and circumference were recorded of the testis and cauda epididymis. Values were recorded for future reference if needed.

Mass motility: For evaluation of mass motility, 25 µL of sperm-Ham's F10 combination was transferred to a pre-warmed (37°C) microscope slide and allowed to stabilise for 2 minutes before motility was recorded. Mass motility was scored using Salamon's system (Evans & Maxwell, 1987), where a score of 1 represented samples with little or no movement, and a score of 5 indicating good progressive motility and a high percentage of motile spermatozoa in the sample.

Concentration determination: The sperm concentration of each sample was determined by using the haemocytometer method (Rouge, 2002). For more details on the dilution, and counting protocol, please refer to Chapter 3, section 3.5.2.

Viability: The nigrosine-eosin vitality stain (Kyron laboratories, South Africa) was used to determine the viability of the epididymal spermatozoa. A droplet of 50µL of staining material was added, and allowed to react with the spermatozoa for 30 seconds. After which a smear was made. An inverted Olympus IX70 microscope fitted with an Olympus SC50 camera was used to analyse a minimum of three different fields using the Olympus cellSense image software (Wirsam, South Africa). Spermatozoa that were stained pink/purple were considered non-viable and non-stained spermatozoa (i.e. white in appearance) were considered to be viable. 200 Spermatozoa were counted per sample, and the total number of live spermatozoa was expressed as a percentage.

Morphology: Abnormal morphologies were counted and recorded using an inverted Olympus IX70 microscope fitted with an Olympus SC50 camera, a minimum of three different fields were observed using the Olympus cellSense image software (Wirsam, South Africa). The morphologies that were analysed were head, midpiece and tail which were included in the total morphology. At least 100 spermatozoa had to be present per slide to calculate the morphology of a smear.

Acrosome Integrity: The acrosome integrity referred to the total percentage of intact acrosomes present on a slide. At least 100 spermatozoa had to be present per slide to calculate the acrosome integrity of a smear. An inverted Olympus IX70 microscope fitted with an Olympus SC50 camera used in conjunction with Olympus cellSense image software (Wirsam, South Africa) was used for the analysis of acrosome integrity. Acrosomes that are

intact will show as a sperm head with a line through the approximate middle section of the head with uniform colouring (Herold *et al.,* 2006).

4.3.6 Statistical analysis

The data analysed and presented in this chapter include data recorded from the adult animals only, with the testis length, width and circumference ranging from 108 to 180mm, 47 to 80mm, and 110 to 231mm, respectively, for the other age categories were represented by too low numbers to warrant analysis and interpretation. The data recorded were analysed with the Microsoft XLStat software program. The total time taken from culling till processing was used as a covariate due to time being a continuous variable. The total time did not have a significant effect on any of the Y variables (namely viability, morphology, acrosome integrity and motility) and thus it was not included in the final model.

Data were transformed according to the transformation that yielded the best results when considering the coefficient of determination, the normality of the residuals as well as the homoscedasticity. The data recorded for the fresh samples were transformed using a log transformation as well as a square root transformation. For the analysis and interpretation of the findings, the log transformation provided a better fit in terms of coefficient of determination, the normality of the residuals as well as the homoscedasticity.

The motility analysis was performed separately from the viability, morphology and acrosome integrity analysis due to not all the motility classes being represented in a proper manner. Microsoft XLStat software was used in order to obtain descriptive statistics on the data.

Statistical analysis indicated an interaction between the treatment and herd, and results are presented in the next section.

4.4 Results

4.4.1 Descriptive statistics

Tables 4.1 and 4.2 represent the testicular and epididymal measurements recorded directly after culling, and after 24h of intact cold storage at 5°C, respectively.

Table 4.1 The average testicular and cauda epididymal measurements (mean ± SD) recorded for African buffalo testes processed immediately after slaughter during a bovine tuberculosis monitoring operation in the Hluhluwe-iMfolozi Game reserve in 2018.

Parameter	Mean ± SD	Range	Coefficient of Variation
Testis length (mm)	135.95 ± 13.45	108.00 – 165.00	0.099
Testis width (mm)	66.62 ± 5.50	55.00 – 76.00	0.083
Testis circumference (mm)	177.24 ± 12.90	145.00 - 200.00	0.073
Epididymis width (mm)	29.14 ± 4.21	15.00 - 35.00	0.145
Epididymis length (mm)	31.52 ± 7.61	20.00 - 46.00	0.241

Table 4.2 The average (mean ± SD) testicular and cauda epididymal measurements recorded for African buffalo testes processed after 24h of cold storage at 5°C during a bovine tuberculosis monitoring operation in the Hluhluwe-iMfolozi Game reserve in 2018.

Parameter	Mean ± SD	Range	Coefficient of Variation
Testis length (mm)	130.45 ± 18.01	110.00 - 180.00	0.138
Testis width (mm)	65.20 ± 6.49	47.00 - 80.00	0.099
Testis circumference (mm)	170.90 ± 22.68	110.00 - 231.00	0.133
Epididymis width (mm)	29.45 ± 3.04	25.00 - 35.00	0.103
Epididymis length (mm)	26.90 ± 6.91	20.00 - 45.00	0.257

Table 4.3 The average (mean ± SD) results recorded for the various parameters included in the study, regardless of the treatment or herd allocation of the African buffaloes during a bovine tuberculosis monitoring operation in the Hluhluwe-iMfolozi Game reserve in 2018.

Parameters	Mean ± SD	Range	Coefficient of variation
Mass motility score (1-5)	1.266 ± 1.087	0 - 5	0.859
Viability (% live)	79.64% ± 11.22%	42.92% - 94.97%	0.141
Morphology (% abnormal)	36.92% ± 15.02%	6.00% - 78.00%	0.407
Head abnormalities (%)	4.23% ± 2.86%	0.00% - 13.00%	0.677
Midpiece abnormalities (%)	21.00% ± 16.24%	0.00% - 78.00%	0.773
Tail abnormalities (%)	11.69% ± 8.84%	0.00% - 40.59%	0.756
Acrosome integrity (%)	12.79% ± 5.84%	1.79% - 27.00%	0.456

4.4.2 The influence of harvesting method and duration of cold storage

The influence of harvesting method and duration of liquid-cold storage on the viability, morphology, and acrosome integrity of African buffalo epididymal spermatozoa are presented in Table 4.4. The residuals for respective parameters were not normally distributed ($P \leq 0.05$) but the variances of these parameters were homoscedastic.

Table 4.4 The influence of harvesting method and liquid cold storage on the viability, morphology and acrosome integrity of epididymal African buffalo spermatozoa collected from adult bulls culled as part of the TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

	Treatment			
Parameter	**0h_0h**	**0h_24h**	**24h_0h**	**24h_24h**
Viability	**85.04%^{A}**	**75.50%^{B}**	**85.14%^{A}**	**75.97%^{B}**
95% Lower bound	80.37%	71.48%	80.47%	71.71%
95% Upper bound	89.98%	79.75%	90.09%	80.49%
Head abnormalities	**4.30%^{A}**	**3.88%^{A}**	**3.52%^{A}**	**3.35%^{A}**
95% Lower bound	2.95%	2.81%	2.50%	2.38%
95% Upper bound	6.27%	5.36%	4.95%	4.71%
Midpiece abnormalities	**19.78%^{A}**	**22.44%^{A}**	**8.58%^{B}**	**21.43%^{A}**
95% Lower bound	13.96%	15.92%	5.86%	14.47%
95% Upper bound	28.02%	31.65%	12.59%	31.73%
Tail abnormalities	**4.48%^{B}**	**8.78%^{AB}**	**9.25%^{A}**	**12.99%^{A}**
95% Lower bound	3.10%	6.17%	6.43%	8.96%
95% Upper bound	6.49%	12.49%	13.31%	18.85%
Total abnormalities	**31.21%^{AB}**	**38.87%^{A}**	**23.53%^{B}**	**41.84%^{A}**
95% Lower bound	25.60%	32.08%	19.29%	34.16%
95% Upper bound	38.06%	47.10%	28.68%	51.25%
Acrosome Integrity	**13.79%^{A}**	**10.88%^{A}**	**10.89%^{A}**	**10.92%^{A}**
95% Lower bound	10.61%	8.44%	8.38%	8.35%
95% Upper bound	17.93%	14.03%	14.16%	14.28%

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$)

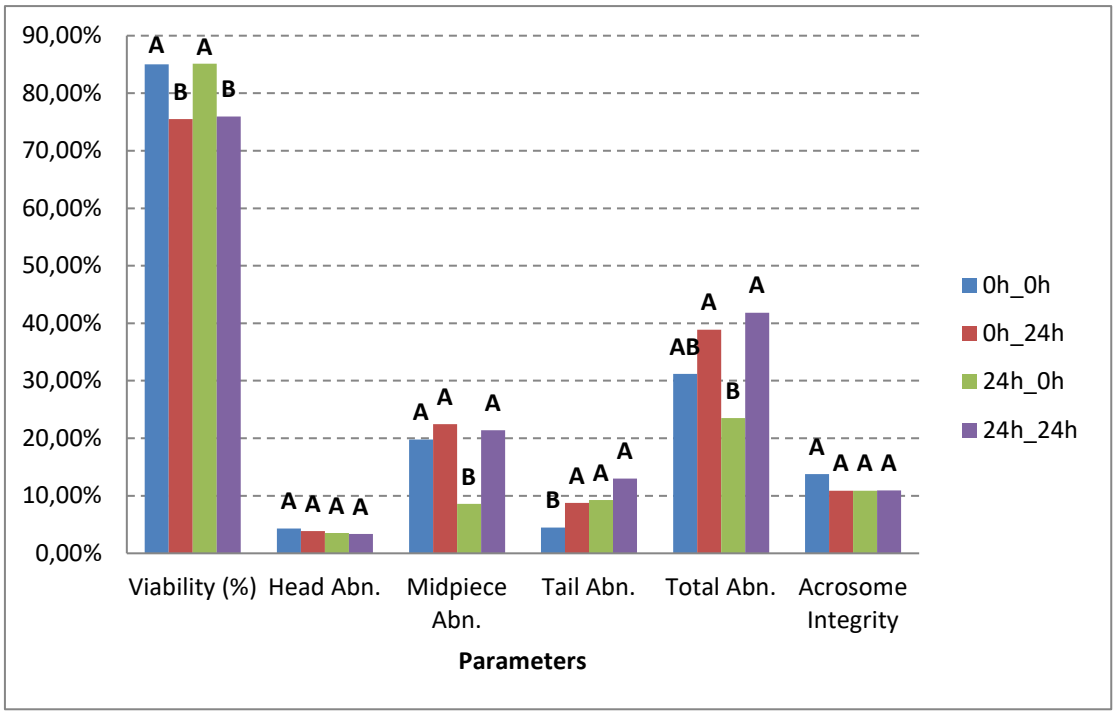

Figure 4.1 The influence of harvesting method and liquid cold storage on the quality parameters of aspirated spermatozoa.

The time that elapsed from aspiration to evaluation influenced sperm viability aspirated from testes stored for 0h and 24h, respectively (Table 4.4). Sperm samples harvested from testes that were processed immediately after collection and analysed immediately post-aspiration, had a higher survival rate than spermatozoa from the same sample set that were stored at 24h at 5°C before evaluation (85.04% vs 75.50%; $P \leq 0.05$). Spermatozoa harvested from testes that were stored for 24h prior to sperm aspiration, similarly demonstrated a higher survival rate than spermatozoa from the same samples stored for 24h at 5°C before analysis (85.14% vs. 75.97%, $P \leq 0.05$). Prolonged storage (i.e. for 24h) of spermatozoa after being removed from the epididymal environment is thus not beneficial in terms of sperm viability, both in cases where testes are processed immediately after culling or when testes are stored intact for 24h after culling before being processed. Survivability of sperm samples aspirated from the epididymal tubules that were processed immediately post-slaughter did not demonstrate a higher survivability when compared to sperm samples aspirated from epididymal tubules after 24h of intact storage at 5°C (85.04% vs. 85.14%, $P > 0.05$). Therefore, the intact storage of testes at 5°C for a prolonged period (i.e. 24h) is a viable alternative to the processing of testes a soon as possible after culling, still yielding a high portion of viable spermatozoa that can be used for *in vitro* fertilisation (IVF) purpose or be processed for long-term cryopreserved storage.

Spermatozoa aspirated from testes after 24h of intact storage at 5°C and analysed immediately demonstrated a lower occurrence of midpiece abnormalities when compared to sperm samples originating from testes that were processed and analysed immediately post-slaughter (8.58% vs. 19.78%; $P \leq 0.05$; Table 4.4). This indicates that intact storage of African buffalo testes at 5°C for a prolonged period of time (i.e. 24h) does not have a deleterious effect on sperm midpiece integrity. Sperm samples aspirated from epididymal tubules of testes that were stored intact at 5°C for 24h before aspiration, had lower levels of midpiece degradation when compared to the same set that were evaluated 24h post-aspiration and when stored at 5° (8.58% vs. 21.43%; $P \leq 0.05$; Table 4.4). Prolonged storage (i.e. no longer than 24h) of aspirated African buffalo epididymal sperm samples at 5°C had a deleterious effect on midpiece integrity, and is thus not advised.

Testes processed post-slaughter with spermatozoa analysed immediately after aspiration had a lower occurrence of tail abnormalities when compared to testes that were stored intact at 5°C for a prolonged period of time (i.e. 24h) regardless of whether these spermatozoa were analysed immediately after aspiration or exposed to 24h of liquid storage at 5°C (4.48% vs. 9.25% and 12.99% respectively; $P \leq 0.05$; Table 4.4). This indicates that tail degradation occurs when sperm samples originating from testes that were stored intact for 24h at 5°C are considered, this negative effect persists during storage of these sperm samples at 5°C post-aspiration (Table 4.4).

Intact testicular cold storage at 5°C for 24h did not lead to the degradation of aspirated sperm samples when considering the overall morphology (i.e. head, midpiece and tail morphology). The degradation of aspirated sperm samples occurred after 24h of liquid storage at 5°C (23.53% vs. 41.84%; $P \leq 0.05$; Table 4.4). Depending on the exposure of the testis to prolonged storage (i.e. 24h) at 5°C the overall morphology was negatively affected by cold storage of aspirated sperm samples. The prolonged storage of testes (i.e. 24h) at 5°C did not affect the morphology of aspirated sperm samples when compared to the same samples that were not exposed to any form of storage (23.53% vs. 31.21%; $P > 0.05$; Table 4.4) and is thus a viable alternative harvesting method (Table 4.4).

The prolonged storage (i.e. 24h) of the testis prior to aspiration or the subsequent storage of aspirated sperm samples at 5°C for 24h did not have an effect on the occurrence of head abnormalities or acrosome integrity (Table 4.4).

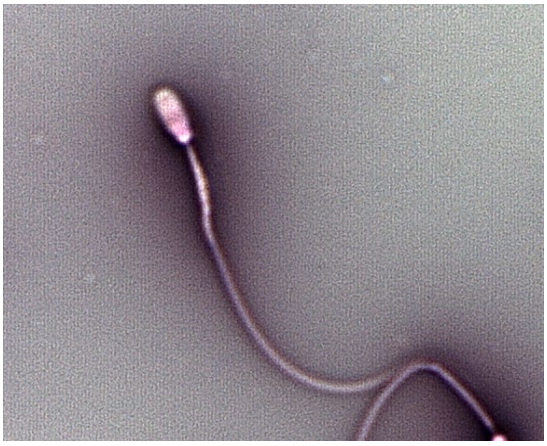

Figure 4.2 The occurrence of a sperm cell with a thickened midpiece in animal B74. The African buffalo testis was processed after prolonged storage (i.e. 24h) at 5°C and sperm samples were analysed immediately post-aspiration.

Figure 4.3 The occurrence of a sperm with a double head and coiled midpiece in animal B29/142. The African buffalo testis was processed immediately post-slaughter and the sperm sample was analysed immediately after

Figure 4.4 The occurrence of a sperm with a split/double tail in animal A66. The African buffalo testis was processed after prolonged storage (i.e. 24h) at 5°C and the sperm sample was analysed immediately after aspiration.

4.4.3 The influence of potential herd effect on the sperm parameters

Three African buffalo herds were captured for determination of the TB status of the herd members. The composition of the respective herds in terms of different age categories and sexes is presented in table 4.5.

Table 4.5 The composition of the three African buffalo herds in terms of age categories and sexes, captured for TB monitoring during 2018 in Hluhluwe-iMfolozi Game Reserve. Although these animals were members of the herds, not all animals were culled due to testing negative for Bovine Tuberculosis.

	Herd A	Herd B	Herd C
Total males	47	20	16
Total females	68	44	38
Total animals	115	64	54
% Male	40.87%	31.25%	29.63%
Mature males	29	12	10
% mature males	25.22%	18.75%	18.52%

The residuals for the viability, head abnormalities, midpiece abnormalities, tail abnormalities, total abnormalities and acrosome integrity were not normally distributed ($P \leq 0.05$) but the variances of these parameters, apart from the tail morphology, were homoscedastic. These results are presented in Table 4.6.

Table 4.6 The influence of herd on the parameters of epididymal African buffalo spermatozoa aspirated from testes collected from adult bulls culled as part of a TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

Parameter	Herd A	Herd B	Herd C
Viability	**80.47%^{A}**	**86.47%^{A}**	**74.35%^{B}**
95% Lower bound	77.00%	81.53%	71.31%
95% Upper bound	84.09%	91.71%	77.53%
Head abnormalities	**2.84%^{A}**	**4.54%^{A}**	**4.07%^{A}**
95% Lower bound	2.18%	3.16%	3.13%
95% Upper bound	3.70%	6.53%	5.28%
Midpiece abnormalities	**23.49%^{A}**	**15.85%^{AB}**	**12.98%^{B}**
95% Lower bound	17.59%	10.71%	10.03%
95% Upper bound	31.36%	23.46%	16.79%
Tail abnormalities	**6.74%^{B}**	**7.52%^{AB}**	**11.26%^{A}**
95% Lower bound	5.05%	5.15%	8.60%
95% Upper bound	8.98%	10.99%	14.73%
Total abnormalities	**35.49%^{A}**	**30.48%^{A}**	**33.40%^{A}**
95% Lower bound	30.41%	24.79%	28.84%
95% Upper bound	41.42%	37.47%	38.67%
Acrosome Integrity	**11.13%^{A}**	**12.70%^{A}**	**10.93%^{A}**
95% Lower bound	9.07%	9.66%	9.00%
95% Upper bound	13.66%	16.69%	13.27%

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$)

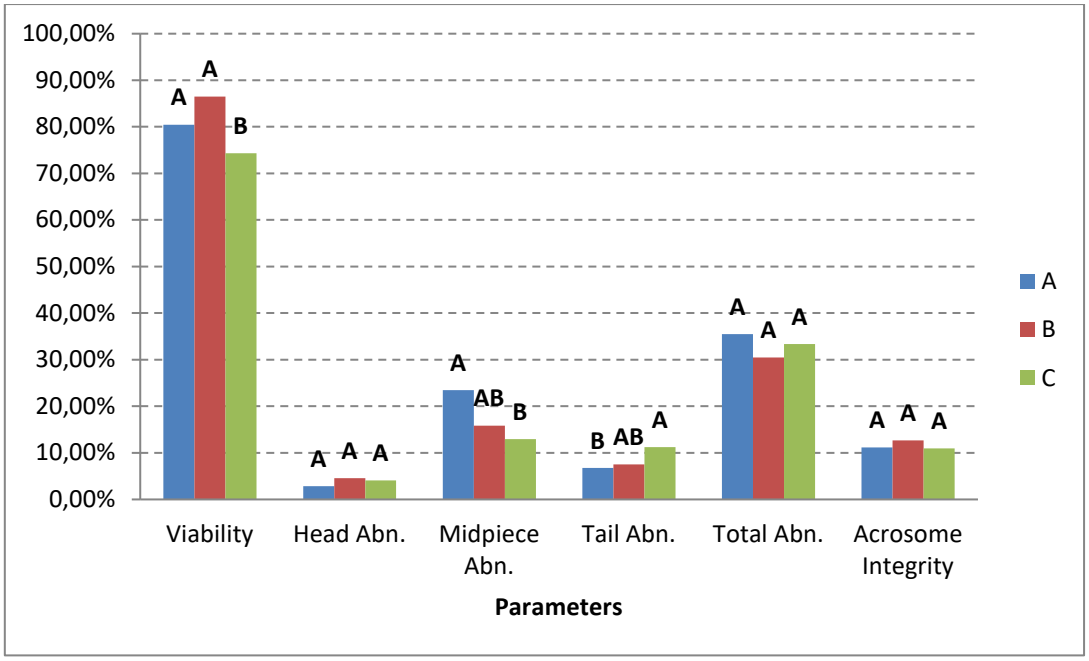

Figure 4.5 The influence of herd on the quality parameters of aspirated spermatozoa. The buffaloes were classified into 3 herds namely A, B and C. Results with different symbols differed significantly (P ≤ 0.05) from one another.

When considering the viability of the sperm samples, regardless of treatments used, sperm samples originating from herds A and B had a higher survivability when compared to samples originating from herd C (80.47% and 86.47% vs. 74.35%; P ≤ 0.05; Table 4.6). Sperm samples originating from Herd C exhibited a lower occurrence of abnormal midpieces when compared to those originating from herd A (12.98% vs. 23.49%; P ≤ 0.05; Table 4.6). Samples from Herd A resulted in lower tail abnormalities when compared to those from herd C (6.74% vs. 11.26%; P ≤ 0.05; Table 4.6). The head morphology, total number of abnormalities occurring and acrosome integrity were not affected by the herds (P > 0.05). The reasoning for the difference in results between the three herds is not known and any reason given would be speculation (Table 4.6).

4.4.4 The interaction between treatment and herd effect

The influence of the interaction between the 4 different treatments and herds on the viability, morphology, and acrosome integrity of African buffalo epididymal spermatozoa are presented in table 4.7. The residuals for respective parameters were not normally distributed ($P \leq 0.05$) but the variances of these parameters were homoscedastic.

Table 4.7 The effect of interaction occurring between herd and the treatment on the parameters of epididymal African buffalo spermatozoa aspirated from testes collected from adult bulls culled as part of a TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

Interaction	Viability	Head abn.	Midpiece abn.	Tail abn.	Total abn.	Acrosome integrity
0h_24h*B	86.05%^{A}	4.75%^{A}	30.95%^{A}	5.65%^{A}	41.85%^{AB}	11.05%^{A}
24h_24h*B	87.26%^{A}	4.59%^{A}	15.39%^{A}	20.89%^{A}	38.59%^{AB}	10.02%^{A}
24h_24h*C	62.87%^{C}	3.66%^{A}	17.94%^{A}	14.81%^{A}	39.91%^{AB}	11.85%^{A}
0h_0h*B	85.09%^{A}	4.49%^{A}	24.00%^{A}	3.41%^{A}	32.50%^{AB}	17.71%^{A}
0h_0h*C	87.07%^{A}	4.90%^{A}	11.64%^{A}	6.51%^{A}	25.03%^{AB}	12.03%^{A}
0h_24h*C	66.18%^{BC}	4.65%^{A}	14.42%^{A}	13.60%^{A}	37.47%^{AB}	10.47%^{A}
24h_24h*A	79.94%^{AB}	2.23%^{A}	35.63%^{A}	7.09%^{A}	47.56%^{A}	10.97%^{A}
0h_0h*A	82.99%^{A}	3.62%^{A}	27.70%^{A}	4.05%^{A}	37.38%^{AB}	12.31%^{A}
24h_0h*B	87.48%^{A}	4.35%^{A}	5.52%^{A}	7.94%^{A}	16.44%^{B}	13.24%^{A}
0h_24h*A	75.58%^{ABC}	2.65%^{A}	25.33%^{A}	8.81%^{A}	37.45%^{AB}	11.14%^{A}
24h_0h*C	84.37%^{A}	3.29%^{A}	9.41%^{A}	12.24%^{A}	33.23%^{AB}	9.57%^{A}
24h_0h*A	83.62%^{A}	3.04%^{A}	12.18%^{A}	8.15%^{A}	23.83%^{AB}	10.20%^{A}

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$)

The viability and total occurrence of abnormalities were affected by the interaction between the treatment applied to the sperm samples and the herd ($P \leq 0.05$; Table 4.7).

The interaction between herd C and sperm samples originating from testes exposed to intact storage and subsequent liquid-storage (both at 5°C for 24h) of aspirated sperm samples resulted in a lower survivability than all other interacting variables (62.87%; $P \leq 0.05$; Table 4.7) apart from herd A and C that interacted with aspirated sperm samples exposed to cold storage (24h) at 5°C processed post-slaughter (75.58% and 66.18%; $P > 0.05$; Table 4.7).

The interaction between herd C and the aspirated sperm samples exposed to cold storage (24h) at 5°C processed post-slaughter resulted in a decreased survivability of the sperm samples when compared to all other interactions that occurred (66.18%, $P \leq 0.05$) apart from herd A and C and the interaction with samples stored (24h) at 5°C prior to aspiration followed by subsequent storage (24h) of aspirated samples (79.94% and 62.87%; $P > 0.05$; Table 4.7) at 5°C as well as the interaction between herd A and aspirated sperm samples stored at 5°C after immediate processing of testes post-slaughter (75.58%; $P > 0.05$; Table 4.7).

The occurrence of abnormal morphology was affected by the interaction between the herd and treatment the sperm was exposed too. The lowest occurrence of abnormal morphology occurred between the interaction of herd B and sperm samples that were exposed to prolonged storage of the testes prior to processing and aspiration, this differed significantly from the interaction between herd A and the prolonged storage (i.e. 24h) of the testis at 5°C prior to processing as well as the storage (24h) of aspirated sperm samples at 5°C (16.44% vs. 47.56%; $P \leq 0.05$; Table 4.7).

4.5 Discussion

The aim of this part of the study was to determine the effect of harvesting method (i.e. immediate processing vs. processing testes after 24h of being stored intact at 5°C) and duration of liquid cold storage (i.e. evaluated immediately after aspiration or after 24h of storage at 5°C) on the mass motility, viability, acrosome integrity, and morphology (i.e. in terms of total morphology, head morphology, midpiece morphology and tail morphology) of epididymal sperm harvested from adult African buffalo testes.

When the lower survival rates of the samples stored for 24h at 5°C, regardless of harvesting method, are considered, it is evident that prolonged exposure to degenerating spermatozoa influenced the survival of the sperm negatively. The poorer survival rate can potentially be ascribed to an accumulation of toxins that are released by the degenerating tissues and cells. Hopkins *et al.* (1988) found that toxins released by degenerating cells can have a toxic effect on spermatozoa. The higher survival rate of spermatozoa evaluated immediately after aspiration and thus that did not require more than one cycle of acclimatization, supports the findings of Murphy *et al.* (2016). In their study, minimising temperature fluctuations resulted in improved sperm survival. Another factor contributing to the decrease in viability after prolonged storage could be the exhaustion of the sperm cell's energy supply. Aspirated sperm samples were diluted with Ham's F10, a cell culture media consisting of sodium bicarbonate and sodium pyruvate, the latter of which acts as an energy substrate within the mixture. The production of ATP within the mitochondria of mammalian spermatozoa occurs through glycolysis which requires the availability of glucose or glycogen in order for the chemical pathway to occur for ATP to be produced (Piomboni *et al.,* 2011; Mukai & Travis, 2012; Ferramosca & Zara, 2014). Thus, although cell culture was added to the sperm samples, over time the substrates required for energy production could have been consumed and thus contributing to a lowered motility for these samples and thus also a lowered survivability (Mukai & Travis, 2012). Some studies indicate that glycolysis is less efficient than oxidative phosphorylation which is a chemical pathway which utilises NADH or $FADH_2$ to create a proton gradient (Piomboni *et al*., 2011). The transmembrane electrical potential that is created due to the proton gradient causes the synthesis of ATP which is utilised as an energy source. However, buffalo species have a lowered uptake of oxygen when compared to other species such as cattle bulls and thus a decrease in motility occurs when samples are exposed to an aerobic pathway such as oxidative phosphorylation when compared to an anaerobic pathway such as glycolysis (Vale *et al*., 2014).

It is evident that cold storage over 24h of aspirated spermatozoa resulted in a decrease in mass motility of the samples. This could be explained by spermatozoa depleting their energy source over time during liquid cold storage and thus having reduced motility (Piomboni *et al.,* 2011; Mukai & Travis, 2012; Ferramosca & Zara, 2014). Despite this, the motility observed was generally quite poor and it is thought that this is due to the spermatozoa being in a quiescent state. Mammals exposed to stressful situations produce an excess of lactate as a result of trying to maintain chemical homeostasis within their bodies (Garcia-Alvarez *et al*., 2014), thus in the case of this trial the buffalo bulls used could have had an increased level of lactate due to being exposed to stressors such as human handling, boma capture and disease (i.e. bovine tuberculosis). This increase in lactic acid leads to cytoplasmic acidification which results in sperm entering a quiescent state and thus exhibiting poorer motility (Matsuzaki *et al*., 2015).

When the effect of harvesting method and duration of liquid cold storage on morphology of the harvested epididymal African buffalo spermatozoa is considered a deterioration of morphological integrity was observed with prolonged storage. It is evident that the total morphology (i.e. sum of head, midpiece and tail abnormalities) of the aspirated sperm samples decreased in quality upon exposure to prolonged storage (i.e. 24h) at 5°C. The intact storage of testis at 5°C however, led to the lowest occurrence of abnormalities when the position on the sperm cell is ignored. This disregards the theory of degenerating cells releasing compounds that are toxic to spermatozoa after 24h of storage at 5°C. It is therefore important to consider the method in which sperm cells will be utilised (i.e. AI, IVF etc.) as the effect and method of storage differs depending on what region of the sperm is most crucial during a specific reproductive technique. These findings were, to our knowledge, unique due to similar studies showing an increase in morphological defects after exposure to intact epididymides storage at various temperatures. A study conducted by Bertol *et al.* (2013) investigated the effect of intact Tabapuã bull epididymis storage at approximately 18-20°C which over time (6-30h) led to an increase in morphological defects.

Tail morphology is one of the most important factors to consider when utilising artificial insemination as part of breeding programmes due to the role of the flagellum in the forward movement of the spermatozoa up through the uterine body and into the Fallopian tubes towards the oocyte. The storage of testes prior to processing resulted in a higher occurrence of tail abnormalities and the occurrence of abnormalities increased during the cold storage of aspirated sperm originating from stored testes. With regards to tail abnormalities it is therefore beneficial to process the testes post-slaughter and, if storage is necessary, the aspirated sperm from these testes can be stored at 5°C up to 24h without major effects. Decreased abnormalities in testes that were processed immediately post-slaughter could be

due to decreased exposure to toxins originating from degenerating cells which has detrimental effects on spermatozoa (Hopkins *et al*. 1988). Minimising temperature fluctuations could also have contributed to the improved results (Murphy et. al., 2016). Another factor to consider is occurrence of chemical imbalances due to the stress experienced by spermatozoa (i.e. increased intact storage, temperature fluctuations etc.) as stress decreases the overall membrane stability of a sperm cell and thus potentially has a negative effect on the tail morphology (Coubrough, 1985; Alejandro *et al*., 2014). These results support the findings of a study conducted by Vilela *et al.* (2017), their research found that the quality of spermatozoa that were not subjected to any form of storage, whether in the testis or liquid cold storage, was higher than the spermatozoa exposed to some form of storage.

To the best of our knowledge there is no information on the effects that the pharmaceutical products used during this study, namely azaperone and diprenorphine, have on the reproductive capabilities and gamete quality of large herbivores.

Sperm samples originating from buffalo's that form part of Herds A and B exhibited higher survivability when compared to those from herd C however, samples from Herd C resulted in a decreased occurrence of midpiece abnormalities. Despite differing parameter results between the three herds there is no indication as to why the results differed due to all management practices, to the best of our knowledge, being identical (all herds were in a 10km radius from one another) as well as the total time that the buffalo herds were within the boma not having a significant effect on the results obtained. A potential explanation could have been the gender composition of the herd; however the bulls only had to service 2.34, 3.67 and 3.8 cows in herds A, B and C respectively. Mature African buffalo bulls (*Syncerus caffer*) that can potentially contribute to the genetic pool usually make up 15% of the herd (Hildebrandt, 2014). This equates to one bull servicing approximately 6.67 cows, and thus the sex ratios seen in the herd composition for this study would not have had a negative impact on the fertility and fecundity of the population.

4.6 Conclusion

Spermatozoa used for artificial insemination and in vitro fertilisation (IVF) can differ slightly in their parameters due to IVF procedures not requiring the same amount of energy or swimming ability due to the spermatozoa being deposited on top of the oocyte, however, the viability as well as the acrosome integrity is equally important in both ART's to ensure optimal fertilisation. It can be concluded that prolonged storage of the testes prior to processing had a negative effect on the tail morphology of the aspirated spermatozoa. Liquid cold storage of spermatozoa post-aspiration had a negative effect on the viability, midpiece morphology and total morphology. Future studies should investigate increasing the liquid-cold storage to 72 hours to see if the possibility of liquid sperm transport would be a viable option for use in breeding programs as well as to determine the fertility of the samples by using methods such as the perivitelline membrane binding assay. Future studies could investigate the effect and possible benefit of storage of the detached epididymis as opposed to storing the entire testis with cauda epididymis intact. The effect of repeated darting with various pharmaceutical products (e.g. M99 and M5050) and maintaining the animals in boma should be considered with regards to the effect on physiological processes (i.e. homeostasis).

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Chapter 5

Influence of Trehalose and thawing rate on the post-thaw viability and survivability of African buffalo (*Syncerus caffer*) spermatozoa

5.1 Abstract

This study investigated the influence of trehalose supplementation as well as thawing rate on the post-thaw viability and survivability of epididymal African buffalo (*Syncerus caffer*) spermatozoa. Sperm samples were aspirated from testes that were either processed immediately after slaughter or stored intact for 24h at 5°C before being processed. The sperm samples were then cryopreserved in either Triladyl® or Triladyl® supplemented with 50mM trehalose. Cryopreserved samples were subsequently thawed at two different rates, i.e. thawing at 80°C for 5 seconds, and thawing at 37°C for 30 seconds, respectively. Parameters recorded included motility, viability, acrosome integrity, and morphology (including total, head, midpiece, and tail morphology). Triladyl® supplemented with trehalose minimised the deleterious effect of cryopreservation for survivability, motility, head morphology, midpiece morphology and acrosome integrity. The only parameter where the effect of cryopreservation was not inhibited was for tail abnormalities, which were more pronounced in the post-thaw samples (2.56% vs. 9.73%, $P \leq 0.05$). The inclusion of trehalose to Triladyl® minimised the deleterious changes that result from cryopreservation, with the latter being evident in a decreased viability (1.76% and 2.32%) and motility (score of 0) reported for epididymal spermatozoa thawed at a fast thawing rate, and that were not cryopreserved in the cryodiluent supplemented with 50mM trehalose. The herd that the animals originated from significantly influenced the survivability and acrosome integrity of epididymal sperm samples, but no definitive trend was identified.

Keywords: Trehalose, African buffalo, spermatozoa, thawing rate, epididymal.

5.2 Introduction

The African buffalo (*Syncerus caffer*) is a member of the so-called Big 5 and is highly sought after in both eco-tourism as well as trophy hunting operations. In 2015, 12% of the total hunting activity earnings, which equates to R145 million, was generated by the hunting of African buffaloes (Coleman, 2018). The African buffalo plays an important role in ecosystems due to this species being a bulk grazer, and in this capacity, it assists in maintaining the grazing quality of veld by utilising the long, fibrous grasses, which in turn create short grasslands that are more suitable for smaller herbivores such as the impala (*Aepyceros melampus*) (Michel & Bengis, 2012, Krugerpark.co.za, 2017).

The African buffalo is susceptible to four economically important diseases namely foot and mouth disease (FMD), Corridor disease, Bovine tuberculosis and Brucellosis (Laubscher & Hoffman, 2012). These diseases have affected the buffalo populations in the Kruger National Park in Mpumulanga as well as Hluhluwe-iMfolozi in Kwa-Zulu Natal (De Vos & van Niekerk, 1969; Laubscher & Hoffman, 2012). Due to the impact of these diseases on the production and reproduction of the African buffalo, collection and processing protocols for the sperm and oocytes of this species needs to be refined to establish a species-specific protocol that can allow for the long term conservation of this species through the preservation of its gametes as part of a national wildlife genome resource bank.

Cryopreservation involves the exposing of biological samples, such as spermatozoa, that are diluted in a suitable medium, to extremely low temperatures to achieve a deep-frozen state in which such samples can be stored for indeterminate periods, if the storage medium (i.e. liquid nitrogen) level is maintained. Liquid nitrogen is the most common substance used for cryopreservation of biological samples, with a storage temperature of -196°C allowing the maintenance of cells in a quiescent state until samples are thawed (Cryogenetics, not dated. Jang *et al*. 2017).

Triladyl® is a TRIS-based cryodiluent that predominantly contains sugar, glycerol and antibiotics and preparation of the working cryosolution requires the addition of egg yolk and distilled water (Minitube, 2018). The main motivation for the inclusion of egg yolk in the cryodiluent is to prevent the occurrence of cold shock during the cryopreservation/thawing process. The exact mechanism how cold shock is prevented is not known, however, it is postulated that the low density lipoproteins in the egg yolk adhere to the cell membranes of spermatozoa, and thus play a role in protecting the spermatozoa against the deleterious effects that result from exposure to low temperatures. Another theory is that spermatozoa's resilience to cold shock is increased due to the egg yolk preventing the loss of membrane phospholipids. In addition to protecting spermatozoa from cold shock, the egg yolk can also

beneficially affect the post-thaw motility of spermatozoa due to its protective properties (Moussa, *et al.*, 2002; Bergeron *et al.,* 2004). In previous studies, Triladyl offered significantly more protection against the deleterious changes caused by the processing and cryopreservation, when compared to AndroMed®, Red Ovine Freezing buffer and Sperm-TALP, when the viability of epididymal African buffalo spermatozoa as well as overall quality are considered (Lambrechts *et al*.,1999; Herold *et al*., 2004; Herold *et al*., 2006).

A compromise in the fertilising ability of spermatozoa can be a direct result of physical and chemical stresses experienced by spermatozoa during collection, processing and cryopreservation, which in turn can result in irreversible changes in cell membrane integrity (Calamera *et al*., 2010; Shah *et al*., 2016). Damage to the cell membrane can be counteracted by adding a supplement to the cryodiluent that confers cryoprotective properties to the cryodiluent supplement mix, assisting in minimising the effect of cryopreservation on sperm viability and function. Glycerol is a common supplement that can be added to cryodiluents to maintain sperm quality; however, glycerol has the potential to become toxic to spermatozoa at certain concentration concentrations, with this toxicity causing a decrease in fertilising ability and viability of spermatozoa (Swelum *et al*., 2011; Iqbal *et al.,* 2018). In a study conducted by Rasul et al. (2007) it was found that glycerol concentration differences affected ejaculated Nili-Ravi buffalo sperm cryopreserved using Trichloroacetic Acid (TCA) extender. A 6% inclusion of glycerol in the cryodiluent resulted in decreased linear motility and increased lateral head displacement when compared to that recorded for samples cryopreserved in a cryodiluent containing 3% glycerol.

Trehalose is a disaccharide that acts as a cryoprotectant during the cryopreservation of sperm cells, and has non-reducing properties. The addition of trehalose to a cryodiluent provides nutrition to the sperm cells during the cryopreservation process (which includes equilibration, cryopreservation as well as thawing and post-thawing), but more importantly plays a protective role in preventing cell membrane damage through maintenance of the osmotic balance and by acting as a membrane stabiliser. Trehalose also prevents ice crystal formation during cryopreservation by dehydrating the spermatozoa, which in turn minimises the deleterious changes that occur during deep freezing at -196°C (Shaikh *et al*., 2016; Zhu *et al*., 2017; Iqbal e*t al.,* 2018). Due to decreased ice crystal formation, it is postulated that trehalose supplementation to cryodiluents will result in an improved viability during short-term liquid and long-term cryopreserved storage, when compared to samples where no supplementation occurs (Tuncer *et al.,* 2013).

The true value of trehalose supplementation potentially lies in trehalose preventing the onset of premature capacitation, which is a crucial component for sperm samples that are used for either artificial insemination (AI) or *in vitro embryo production* (IVEP) (Shaikh *et al*., 2016. Zhu *et al*., 2017; Iqbal e*t al.,* 2018). In a study conducted on Kankrej bull semen, the addition of trehalose improved post-thaw motility, acrosomal integrity as well as plasma membrane integrity of the bull spermatozoa (Shaikh, *et al.*, 2016).

To the best of our knowledge, there is no literature available on studies that investigated the potential of trehalose in African buffalo sperm cryodiluents to minimise the deleterious changes caused by processing and cryopreservation.

The time that spermatozoa are exposed to cryodiluents prior to cryopreservation is referred to as the equilibration period. This period not only allows for the spermatozoa to adapt to the colder temperature over a period of time in order to prevent temperature shock, but also served to prevent chemical shock of the spermatozoa once they are introduced to a cryodiluent prior to cryopreservation, which in turn will allow for the gradual displacement of water from the spermatozoa (Shah *et al.*, 2016). The timing of the equilibration period is thus vital to the successful survival of the spermatozoa post-thaw. Herold *et al.* (2004) conducted a study that compared different equilibration periods when cryopreserving epididymal African buffalo spermatozoa using different cryodiluents. The study concluded that an equilibration time of between 2 to 9 hours is recommended, as this resulted in the lowest percentage of sperm mortalities.

The most common procedure used for the cryopreservation of animal sperm is characterised by three important stages. The first stage includes equilibration which prevents chemical shock as well as exposes the spermatozoa to a gradual decrease in temperature. Under normal procedures spermatozoa are equilibrated at approximately 4°C for around 2-3 hours in order to gradually decrease the temperature. This is then followed by loading of the French Cassou straws (i.e. semen straws) and these straws are subsequently suspended in the liquid nitrogen vapour for 10-15 minutes (temperature between -140°C and -180°C) before being plunged directly into the liquid nitrogen (-196°C) for indefinite storage (Lambrechts *et al.,* 1999; Herold *et al*., 2004; Bertol, 2016; Iqbal *et al*., 2018). This method was successfully used by Lambrechts *et al*. (1999) on African buffalo epididymal sperm samples exposed to cryopreservation as well as Herold *et al*. (2004) who investigated the effect that different cryodiluents had on the survival of post-thaw epididymal African buffalo spermatozoa.

Thawing temperature refers to the temperature that semen/sperm straws are thawed at. The thawing temperature plays an important role in maintaining the cell membrane integrity as well as controlling the osmotic shock that spermatozoa might experience. In a study conducted by Correa *et al*. (1996) various thawing and processing temperatures of bovine spermatozoa were evaluated and it was concluded that a thawing temperature of 37°C for a minimum of 30 seconds resulted in the best motility results. For optimal quality the Correa et al. study thawed sperm at 37°C and processed the sperm post-thaw at a temperature of 21°C. Similar results were obtained in a study of Shah *et al.* (2016) on water buffalo (*Bubalus bubalis*) where various thawing temperatures and times were investigated. Shah *et al*. (2016) concluded that thawing at 37°C for 30 seconds resulted in improved sperm quality than when samples were thawed at 30°C and 75°C for 30 and 9 seconds respectively. In a study conducted by Lambrechts (1996), two thawing rates were used to thaw cryopreserved epididymal African buffalo spermatozoa. The thawing rates were 35°C for 30 seconds and 80°C for 5 seconds, and it was found that a fast thawing rate did not yield improved motility results when compared to the standard thawing rate at 35°C.

Cryopreservation is known to decrease the fertility of spermatozoa due to damage to the sperm plasma membrane as well as a change in membrane permeability, which can eventually lead to premature capacitation, i.e. before reaching the zona pellucida of the oocyte (Talukdar *et al.,* 2017). It is of vital importance to prevent the onset of capacitation before the spermatozoa are in close proximity to the oocyte in the female reproductive tract during natural mating or AI, or alternatively using *in-vitro* fertilisation during IVEP protocols. Premature capacitation leads to the inability for spermatozoa to successfully fertilise the oocyte, and once capacitated, death of spermatozoa occurs naturally as a result of the destabilisation of the surface membrane (Cormier *et al*., 1997; Ickowicz *et al.,* 2012). Once capacitation occurs spermatozoa experience an increased level of membrane permeability which leads to increased intake of calcium ions thus resulting in the initiation of the acrosome reaction (Cormier *et al*., 1997; Ickowicz *et al.,* 2012). Due to this reaction occurring prior to reaching the oocyte premature capacitation results in decreased fertility as the acrosin and hyaluronidase required to ensure penetration of the zona pellucida, will have been released prematurely, rendering the sperm incapable of participating in fertilisation (Cormier *et al*., 1997; Ickowicz *et al.,* 2012; Vale *et al.,* 2014).

The aim of this study was therefore to determine the influence of trehalose supplementation and thawing rate on the mass motility, survivability, morphology and acrosome integrity of cryopreserved epididymal African buffalo spermatozoa obtained from testes of bulls that were culled as part of a TB monitoring program.

5.3 Methodology

5.3.1 Experimental Location

The collection of samples took place in the Hluhluwe-iMfolozi Game Reserve during the 2018 Bovine Tuberculosis monitoring operation. The reserve is located in the northern part of the Kwa-Zulu Natal Province, and is characterized by a humid, subtropical climate. This region experiences hot, wet summers and mild, dry winters, with an average annual temperature of 19.5°C and an average annual rainfall of 957mm (Climate-data.org, 2019).

Post-thaw evaluation was carried out in the Animal Physiology laboratory of the Department of Animal Sciences of Stellenbosch University, Stellenbosch, Western Cape. This area is characterized by a Mediterranean climate and experiences cool, wet winters and hot, dry summers. This area is characterised by an average annual temperature of 16.4°C and an annual rainfall of 802mm (Climate-data.org, 2019).

5.3.2 Experimental animals and collection of testes

Testes were collected from African buffalo (Syncerus caffer, N=114) that were culled using a rifle shot as part of a Bovine Tuberculosis monitoring program (with animals testing positive for bovine tuberculosis being eradicated) carried out in the Hluhluwe-iMfolozi Game Reserve, animals culled consisted of both sexes. Testes were collected from 26 adolescent and mature bulls with 21 mature bulls contributing to this study, due to the adolescent males not producing functioning sperm. Three individual herds (i.e. Herd A, Herd B and Herd C) that consisted of adults, young adults, sub adults, juveniles and calves of both sexes, were culled in the period 17/07/2018 to 02/08/2018. Herds A and B were captured in the same location on the Reserve, and Herd C was captured 8.2km from this location. Prior to culling the animals were maintained in a boma to facilitate sedation for blood sampling and skin tests to determine the bovine tuberculosis status as well as the extent of infection.

The pharmaceutical products used to achieve sedation included Stresnil (azaperone-M99, Sigma-Aldrich) for sedation as well as M5050 (diprenorphine (Sigma-Aldrich) to reverse the sedation. Care was taken to ensure that all animals had *ad libitum* access to feed and fresh drinking water during the holding period. Herds A, B and C were maintained in the boma between 16 to 25 days, 15 to 22 days and 10 to 19 days, respectively.

5.3.3 Experimental design

The trehalose supplementation experiment was a 2x2 factorial design and the thawing rate experiment was a 4x2 factorial design. Treatments included two different trehalose concentrations namely 0mM and 50mM and two different thawing rates namely at 37°C for

35 seconds and 80°C for 5 seconds. The first value of the treatment label indicates whether the testis was processed immediately after slaughter (i.e. 0 hours intact) or whether the testis was subjected to prolonged storage at 5°C for 24 hours prior to processing (i.e. 24 hours intact). The second (or middle value) symbolised the temperature at which the samples were thawed, either for 35 seconds at 37°C or for 5 seconds at 80°C. The last part of the treatment labels symbolises whether the spermatozoa were cryopreserved in the cryodiluent only (i.e. Tri) or whether the cryodiluent was supplemented with trehalose (i.e. T+T).

5.3.4 Processing of testes and samples

For allocation to the respective treatment groups, the testes of each bull were collected, and from each pair, one testis was processed immediately after culling, whereas the other testis was stored intact and protected against dehydration, at 5°C for 24h before being processed.

Processing of each testes and aspirated sperm samples were carried out at room temperature (about 22°C).The *tunica albuginea* of each testis was cut open to expose the testis and epididymis. After removal of the *tunica albuginea,* testis length, width and circumference were recorded. The epididymal dimensions (i.e. length and width) were recorded prior to epididymal processing. The cauda epididymis including a part of the *vas deferens* was then dissected free from the testis, and transferred to an unused 90mm Petri dish, and care was taken to prevent dehydration of the epididymis.

To obtain the epididymal sperm samples, the tubules of each cauda epididymis were exposed by removing the connective tissue and blood vessels using a scalpel blade (size no. 11, lasec) and a pincette. After exposure, a large portion of the tubules before transition into the vas deferens was removed and transferred to a 65mm Petri dish containing 3mL Ham's-F10 (Sigma-Aldrich) prewarmed to 37°C (Lambrechts *et al*., 1999; Herold *et al*., 2006). A slicing method was used to allow for the release of the spermatozoa into the collection medium (Bertol, 2016). Once slicing was completed the tubules were rinsed with another 1mL Ham's F-10 (pre-warmed to 37°C), to ensure optimal sperm recovery. The Petri dish containing the epididymal spermatozoa and Ham's F-10 nutrient combination was then placed on a warming plate (MH6616, Electrothermal) set at 37°C to prevent temperature fluctuations.

5.3.5 Dilution of samples

The amount of straws of epididymal spermatozoa that would be cryopreserved was dependent on the concentration as well as the volume of overall sample available. The following formulae were used to calculate the dilution of each raw sample to obtain an end concentration of 30 million spermatozoa per millilitre:

- $n\ of\ straws\ desired\ x\ 0.25ml = total\ v\ needed\ (in\ ml)$
- $\frac{desired\ spermatozoa\ concentration}{actual\ spermatozoa\ concentration} = v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v$
- $v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v\ x\ total\ ml\ needed = total\ v\ of\ spermatozoa\ mixture\ needed$
- $Total\ v\ needed - total\ v\ of\ spermatozoa\ mixture\ needed = v\ of\ cryodiluent\ needed$

For more details refer to section 3.5.5.1.

5.3.6 Cryopreservation and equilibration of spermatozoa

Once the epididymal spermatozoa and Ham's F-10 nutrient-rich mixture was diluted with the respective pre-warmed (37°C) cryodiluent by gradually adding the respective diluents using a calibrated pipette to prevent chemical shock to the epididymal spermatozoa. The 15 mL Falcon tubes were gently everted 2-3 times and transferred to a 250mL glass beaker containing H_2O (room temperature) and then equilibrated at 5°C for approximately 3 to 4 hours (Lambrechts *et al*., 1999; Herold *et al*., 2006). At this point the 0.25cc French straws that would be used for the cryopreservation of the spermatozoa samples were marked clearly (information included animal ID, date, sample treatment) and placed in the refrigerator at 5°C in order to reduce the risk of temperature shock to the spermatozoa once the loading of the French straws commenced. After the 3-4 hours of equilibration the spermatozoa/cryodiluent mixtures were loaded into the labelled 0.25cc French straws. The French straws' end without the cotton wool plug was then sealed off using PVC powder. Once the French straws were loaded and sealed they were suspended in liquid nitrogen vapour (-140 to -180°C) for 15 minutes, and then submerged directly into the liquid nitrogen (at -196°C) for 5 minutes (Herold *et al*., 2006; Swelum *et al*., 2011) before transferring them into the canes and goblets of the liquid nitrogen cryopreservation tank. Location of each sample set was recorded for future reference purposes.

5.3.7 Thawing of cryopreserved straws

Cryopreserved samples were subjected to two different thawing rates. The first treatment consisted of thawing the epididymal spermatozoa straws for 30 seconds in a waterbath maintained at 37°C (to ensure no temperature fluctuations occurred during thawing) (Swelum *et al*., 2011) before emptying the sample into a 1ml Eppendorf tube. The same procedures were followed with the second treatment, however, the cryopreserved sperm straws were thawed at 80°C for 5 seconds (Lambrechts *et al.,* 1999).

5.3.8 Data recorded

Testis and cauda epididymis measurements: Measurements of the length, width and circumference were recorded of the testis and cauda epididymis. Values were recorded for future reference if needed.

Mass motility: For evaluation of mass motility, 25 µL of sperm-Ham's F10 combination was transferred to a pre-warmed (37°C) microscope slide and allowed to stabilise for 2 minutes before motility was recorded. Mass motility was scored using Salamon's system (Evans & Maxwell, 1987), where a score of 1 represented samples with little or no movement, and a score of 5 indicating good progressive motility and a high percentage of motile spermatozoa in the sample.

Concentration determination: The sperm concentration of each sample was determined by using the haemocytometer method (Rouge, 2002). For more details on the dilution, and counting protocol, please refer to Chapter 3, section 3.5.2.

Viability: The nigrosine-eosin vitality stain (Kyron Laboratories, South Africa) was used to determine the viability of the epididymal spermatozoa. A droplet of 50µL of staining material was added, and allowed to react with the spermatozoa for 30 seconds, after which a smear was made. An inverted Olympus IX70 microscope fitted with an Olympus SC50 camera, was used to analyse a minimum of three different fields using the Olympus cellSense image software (Wirsam, South Africa). Spermatozoa that were stained pink/purple were considered non-viable and non-stained spermatozoa (i.e. white in appearance) were considered to be viable. A minimum of 200 spermatozoa were counted per sample, and the total number of live spermatozoa was expressed as a percentage.

Morphology: Abnormal morphology was evaluated using an inverted Olympus IX70 microscope fitted with an Olympus SC50 camera. A minimum of three different fields per slide were observed using the Olympus cellSense image software (Wirsam, South Africa). Sperm regions analysed included the head, midpiece and tail, with all then expressed as

total percentage normal morphology. A minimum of 200 spermatozoa had to be present per slide to calculate the morphology of a smear.

Acrosome Integrity: Acrosome integrity refers to the total percentage of intact acrosomes in a particular sample. An inverted Olympus IX70 microscope fitted with an Olympus SC50 camera used in conjunction with Olympus cellSense image software (Wirsam, South Africa) was used for the analysis of acrosome integrity. Acrosomes that are intact will show as a sperm head with a line through the approximate middle section of the head with uniform colouring (Herold *et al.,* 2006). A minimum of 200 spermatozoa had to be present per slide to calculate the acrosome integrity of a smear.

5.3.9 Statistical analysis

The data analysed and presented in this chapter include data recorded from the adult animals only, with the testis length, width and circumference ranging from 108 to 180mm, 47 to 80mm, and 110 to 231mm, respectively, for the other age categories were represented by too low numbers to warrant analysis and interpretation. The data recorded were analysed with the Microsoft XLStat software program. The total time taken from culling till processing was used as a covariate due to time being a continuous variable. The total time did not have a significant effect on any of the Y variables (namely viability, morphology, acrosome integrity and motility) and thus it was not included in the final model.

The data set for the cryopreserved samples underwent a log transformation due to it yielding better results with regards to the coefficient of determination, normality of the residuals as well as the homoscedasticity of the variances. The motility analysis was performed separately from the viability, morphology and acrosome integrity analysis due to not all the motility classes being represented in a proper manner. Microsoft XLStat software was used in order to obtain descriptive statistics on the motility data. No interaction took place in the log transformed data set for the cryopreserved data and therefore we could consider the data without looking at the effect of interaction.

The cryopreserved samples were compared to the fresh samples to determine whether cryopreservation had a significant effect on the quality of aspirated sperm samples when compared to samples originating from the same testis that were not subjected to cryopreservation. The data were transformed using a square root transformation due to it having better results with regards to normality and homoscedasticity than when compared to the non-transformed data as well as the log transformed data. Data were converted back to normal values before being represented. Descriptive statistics will not be discussed in this section due to this analysis having been discussed in previous sections for both the fresh as

well as cryopreserved samples. During this comparison interaction occurred between the variables and could thus not be ignored. To determine whether there was a significant difference between the parameters before and after cryopreservation occurred a paired T test was carried out on the combined data.

5.4 Results

5.4.1 Descriptive statistics

Table 5.1 represents the descriptive statistics for the respective sperm parameters recorded during the study. The largest degree of variation was recorded for mass motility (1.199), and the lowest degree of variation was recorded for the total percentage of abnormal morphology (0.377).

Table 5.1 African buffalo (Syncerus caffer) epididymal sperm parameters (mean ± SD) recorded recorded during a bovine tuberculosis monitoring operation in the Hluhluwe-iMfolozi Game reserve in 2018.

Parameters	Mean ± SD	Range	Coefficient of variation
Mass motility score (1-5)	0.513 ± 0.615	0.00 – 3.00	1.199
Viability (% live)	8.27% ± 0.57%	0.00% - 31.02%	0.914
Morphology (% abnormal)	47.45% ± 17.90%	3.90% - 85.14%	0.377
Head abnormalities (%)	3.15% ± 2.03%	0.00% - 9.00%	0.645
Midpiece abnormalities (%)	35.14% ± 16.14%	2.91% - 68.62%	0.459
Tail abnormalities (%)	9,16% ± 9,04%	0.00% - 46.53%	0.986
Acrosome integrity (%)	8.87% ± 7.03%	0.97% - 35.00%	0.793

5.4.2 The influence of trehalose supplementation and thawing rate

The influence of trehalose supplementation and different thawing rates on the viability, morphology and acrosome integrity of African buffalo spermatozoa are presented in Table 5.2. The residuals for the midpiece morphology, total abnormal morphology and acrosome integrity were not normally distributed ($P \leq 0.05$). The variances of all the parameters, namely viability, morphology (including head, midpiece and tail), and acrosome integrity were homoscedastic ($P > 0.05$).

Table 5.2 The influence of trehalose supplementation and thawing rate on the viability, morphology and acrosome integrity of epididymal African buffalo spermatozoa collected from adult bulls culled as part of the TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

	Treatment							
Parameter	**0h_37_Tri**	**0h_37_T+T**	**24h_37_Tri**	**24h_37_T+T**	**0h_80_Tri**	**0h_80_T+T**	**24h_80_Tri**	**24h_80_T+T**
Viability	**12,67%^{AB}**	**18,78%^{A}**	**7,09%^{ABC}**	**9,82%^{AB}**	**1,76%^{D}**	**7,02%^{ABC}**	**2,32%^{CD}**	**4,07%^{BCD}**
95% Lower bound	8,05%	11,74%	4,00%	5,31%	1,08%	4,31%	1,25%	2,19%
95% Upper bound	19,94%	30,02%	12,56%	18,16%	2,87%	11,44%	4,29%	7,57%
Head Morpholog	**2,28%^{A}**	**2,82%^{A}**	**3,11%^{A}**	**2,52%^{A}**	**2,40%^{A}**	**3,08%^{A}**	**3,03%^{A}**	**3,58%^{A}**
95% Lower bound	1,56%	1,90%	1,93%	1,50%	1,59%	2,04%	1,81%	2,13%
95% Upper bound	3,33%	4,17%	5,02%	4,21%	3,61%	4,63%	5,06%	6,02%
Midpiece Morpholog	**24,18%^{A}**	**31,25%^{A}**	**25,42%^{A}**	**36,89%^{A}**	**27,17%^{A}**	**33,93%^{A}**	**30,02%^{A}**	**36,45%^{A}**
95% Lower bound	16,79%	21,42%	16,04%	22,49%	18,34%	22,91%	18,30%	22,11%
95% Upper bound	34,83%	45,60%	40,28%	60,49%	40,24%	50,26%	49,26%	60,08%
Tail Morpholog	**3,42%^{BC}**	**7,80%^{ABC}**	**9,01%^{ABC}**	**13,50%^{A}**	**2,56%^{C}**	**9,73%^{AB}**	**7,64%^{ABC}**	**10,99%^{AB}**
95% Lower bound	2,11%	4,73%	4,90%	7,01%	1,52%	5,78%	3,97%	5,67%
95% Upper bound	5,54%	12,86%	16,58%	25,99%	4,30%	16,37%	14,73%	21,31%
Total Morpholog	**33,61%^{A}**	**45,51%^{A}**	**39,71%^{A}**	**57,08%^{A}**	**34,35%^{A}**	**51,28%^{A}**	**42,26%^{A}**	**55,15%^{A}**
95% Lower bound	25,79%	34,60%	28,43%	39,86%	25,835	38,56%	29,50%	38,37%
95% Upper bound	43,79%	59,87%	55,45%	81,72%	45,68%	68,20%	60,53%	79,26%
Acrosome Integrity	**5,91%^{A}**	**4,88%^{A}**	**4,94%^{A}**	**5,99%^{A}**	**5,53%^{A}**	**4,33%^{A}**	**3,54%^{A}**	**4,60%^{A}**
95% Lower bound	3,54%	2,87%	2,59%	2,99%	3,19%	2,49%	1,76%	2,28%
95% Upper bound	9,86%	8,30%	9,42%	12,00%	9,60%	7,52%	7,09%	9,29%

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$)

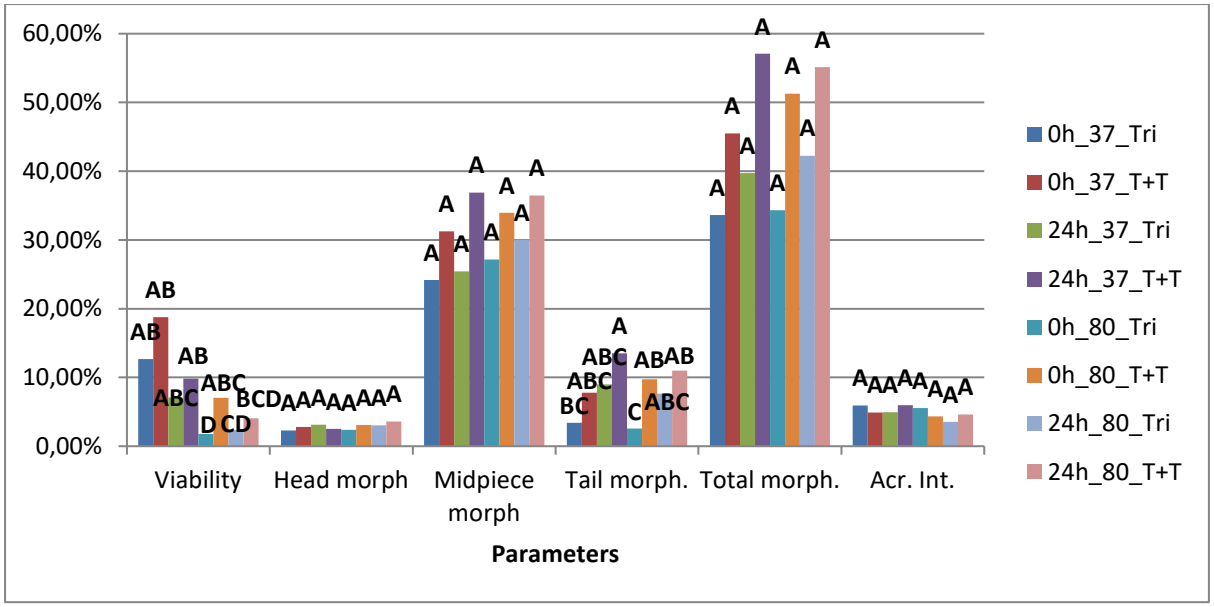

Figure 5.1 The influence of trehalose supplementation and thawing rate on the quality parameters of aspirated cryopreserved spermatozoa.

The prolonged storage (i.e. 24h) of the testes at 5°C reduced the survivability of cryopreserved samples. Spermatozoa samples aspirated from testes immediately post-slaughter and cryopreserved in the cryodiluent supplemented with 50mM trehalose and thawed at 37°C for 35 seconds exhibited a higher survivability than samples subjected to prolonged storage at 5°C prior to sperm aspiration and thawed at 80°C for 5 seconds, regardless of whether the samples were supplemented with trehalose or not (18.78% vs. 2.32% and 4.07%; $P \leq 0.05$; Table 5.2). Therefore, prolonged storage (i.e. 24h) of testis prior to aspiration for cryopreservation purposes combined with a fast thawing rate is not a suitable method for the processing of African buffalo spermatozoa.

Spermatozoa samples aspirated from testes immediately post-slaughter and cryopreserved in the cryodiluent supplemented with 50mM trehalose and thawed at 37°C for 35 seconds also exhibited a higher survivability when compared to samples originating from testes that were processed immediately post slaughter but exposed to a fast thawing rate in the absence of trehalose (18.78% vs. 1.76%; $P \leq 0.05$; Table 5.2). There was, however, no difference between the former treatment and the survivability of spermatozoa that were exposed to a fast thawing rate in the presence of trehalose (18.78% vs. 7.02%, $P > 0.05$). This suggests that trehalose provides a protective role to the spermatozoa in terms of the survivability of aspirated samples.

Sperm samples that were thawed at 37°C for 35 seconds with differing pre-cryopreservation processes as well as supplementation differences exhibited a higher survivability than sperm samples thawed at 80°C for 5 seconds with no trehalose supplementation (12.67% and

9.82% vs. 2.32% and 1.76%; P ≤ 0.05; Table 5.2). A fast thawing rate thus negatively impacted on the survivability of cryopreserved sperm samples, particularly in the absence of trehalose. The importance of trehalose supplementation for the survivability of aspirated sperm samples is evident in samples exposed to the same processing and thawing methods differing in survivability due to the supplementation of trehalose pre-cryopreservation (7.02% vs. 1.76%; P ≤ 0.05; Table 5.2).

Sperm samples exposed to a prolonged storage (i.e. 24h) at 5°C prior to aspiration and subsequently supplemented with trehalose before being thawed at 37°C for 35 seconds showed a higher occurrence of tail abnormalities when compared to sperm samples aspirated post-slaughter without the addition of trehalose, regardless of the thawing rate (13.50% vs. 2.56% and 3.42%, P ≤ 0.05). This indicates that trehalose has a negative effect on the tail morphology of aspirated sperm samples. This is confirmed by the increased occurrence of tail abnormalities in samples supplemented with trehalose, despite the thawing rate (80°C for 5 seconds) and the time elapsed between slaughter and aspiration being equal (2.56% vs. 9.73%; P ≤ 0.05; Table 5.2).

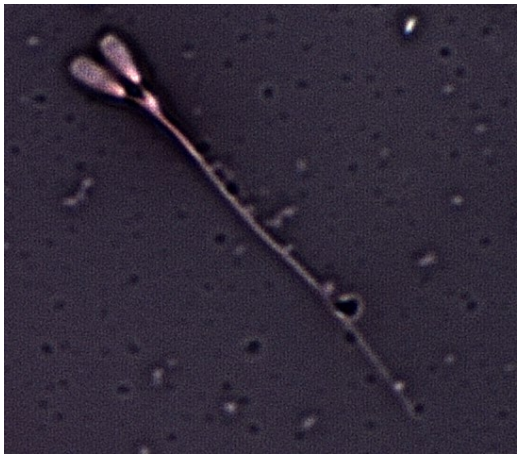

Figure 5.2 The occurrence of a sperm with a double head in animal A66. The sample was subjected to immediate processing post-slaughter and was not exposed to trehalose supplementation. Thawing occurred at 80°C for 5 seconds.

Figure 5.3 The occurrence of a sperm with a double head in animal C76. The sample was subjected to prolonged storage at 5°C prior to processing. The sperm sample was supplemented with 50mM trehalose pre-equilibration and was thawed at 37°C for 35 seconds.

5.4.3 The influence of a potential herd effect on the sperm parameters

Three African buffalo herds were identified and captured for determination of the TB status of the herd members. The composition of the respective herds in terms of different age categories and sexes is presented in Table 5.3.

Table 5.3 The composition of the three African buffalo herds in terms of age categroies and sexes, captured for TB monitoring during 2018 in Hluhluwe-iMfolozi Game Reserve. Although these animals were members of the herds, not all animals were culled due to testing negative for Bovine Tuberculosis.

	Herd A	Herd B	Herd C
Total males	47	20	16
Total females	68	44	38
Total animals	115	64	54
% Male	40.87%	31.25%	29.63%
Mature males	29	12	10
% Mature males	25.22%	18.75%	18.52%

The residuals for the midpiece abnormalities, total abnormalities and acrosome integrity were not normally distributed ($P \leq 0.05$). The variances of all the parameters, namely viability, morphology (including head, midpiece and tail), and acrosome integrity were homoscedastic ($P > 0.05$). These results are presented in Table 5.4.

Table 5.4 The influence of herd on the parameters of epididymal African buffalo spermatozoa aspirated from testes collected from adult bulls culled as part of a TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

	Herd		
Parameter	**A**	**B**	**C**
Viability	**5,86%^{AB}**	**9,11%^{A}**	**4,26%^{B}**
95% Lower bound	4,32%	5,39%	3,29%
95% Upper bound	7,95%	15,41%	5,53%
Head Morphology	**3,08%^{A}**	**2,35%^{A}**	**3,10%^{A}**
95% Lower bound	2,38%	1,52%	2,50%
95% Upper bound	3,97%	3,65%	3,86%
Midpiece Morphology	**26,71%^{A}**	**29,90%^{A}**	**34,91%^{A}**
95% Lower bound	20,90%	19,59%	28,32%
95% Upper bound	34,14%	45,64%	43,03%
Tail Morphology	**8,81%^{A}**	**6,29%^{A}**	**6,58%^{A}**
95% Lower bound	6,37%	3,59%	4,99%
95% Upper bound	12,19%	11,01%	8,68%
Total Morphology	**43,77%^{A}**	**40,54%^{A}**	**48,23%^{A}**
95% Lower bound	36,63%	29,83%	41,44%
95% Upper bound	52,29%	55,11%	56,14%
Acrosome Integrity	**6,26%^{AB}**	**2,81%^{C}**	**6,68%^{A}**
95% Lower bound	4,44%	1,55%	4,98%
95% Upper bound	8,84%	5,09%	8,97%

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$).

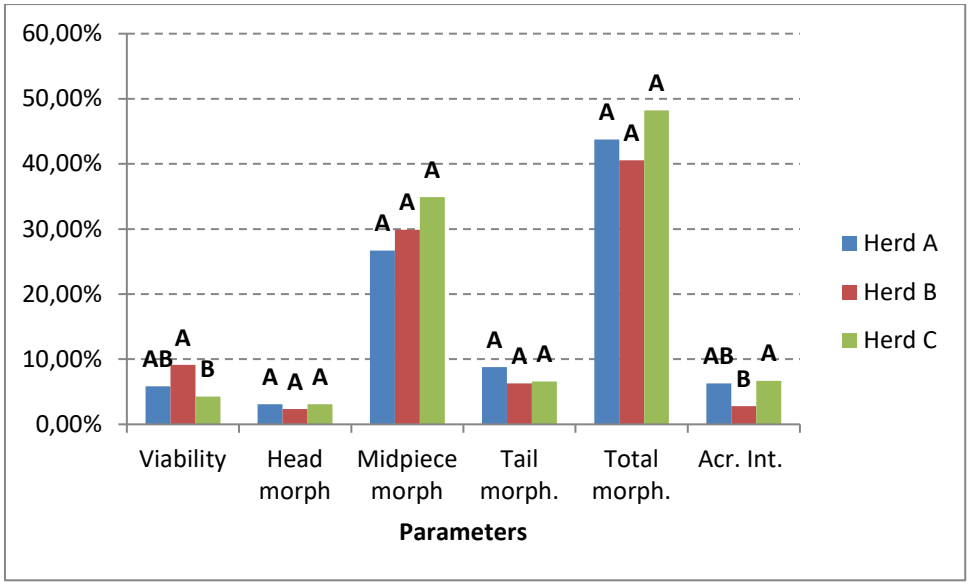

Figure 5.4 The influence of herd on the quality parameters of aspirated spermatozoa. The buffaloes were classified into 3 herds namely A, B or C. Results with different symbols differed significantly (P ≤ 0.05) from one another.

When considering the survivability of aspirated sperm samples, regardless of the treatment used, spermatozoa obtained from Herd B bulls demonstrated a higher survivability when compared to Herd C (9.11% vs. 4.26%; P ≤ 0.05; Table 5.4). Herd C had a higher occurrence of intact acrosomes in the sperm samples when comparing the results to Herd B (6.68% vs. 2.81%; P ≤ 0.05; Table 5.4). The reasoning for the difference in results between the three herds is not known and any reason given would be speculation (Table 5.4).

5.4.4 Comparison of fresh and cryopreserved samples

The fresh samples were compared to the cryopreserved samples in order to determine the overall effect of cryopreservation (Table 5.5).

Table 5.5 The influence of cryopreservation on aspirated sperm samples when compared to the same samples pre-cryopreservation and the effect on the viability, morphology, and acrosome integrity of epididymal African buffalo spermatozoa collected from adult bulls culled as part of the TB monitoring operation during 2018 in the Hluhluw-iMfolozi Game Reserve.

	Treatment									
Parameter	**0h_0h**	**24h_0h**	**0h_37 Tri**	**0h_37 T+T**	**24h_37 Tri**	**24h_37 T+T**	**0h_80 Tri**	**0h_80 T+T**	**24h_80 Tri**	**24h_80 T+T**
Viability	**85,19%^{A}**	**85,25%^{A}**	**13,69%^{B}**	**14,31%^{B}**	**10,52%^{BC}**	**12,39%^{B}**	**1,37%^{E}**	**7,67%^{BCD}**	**2,67%^{DE}**	**3,80%^{CDE}**
95% Lower bound	78,76%	78,82%	9,72%	10,24%	6,88%	8,22%	0,35%	4,78%	1,03%	1,68%
95% Upper bound	91,87%	91,94%	18,34%	19,06%	14,94%	17,42%	3,07%	11,24%	5,09%	6,77%
Head Morph.	**3,08%^{A}**	**3,61%^{A}**	**2,56%^{A}**	**2,27%^{A}**	**3,02%^{A}**	**2,00%^{A}**	**2,60%^{A}**	**3,22%^{A}**	**3,51%^{A}**	**2,64%^{A}**
95% Lower bound	1,90%	2,32%	0,96%	0,79%	1,17%	0,52%	0,99%	1,38%	1,48%	0,87%
95% Upper bound	4,54%	5,18%	4,92%	4,51%	5,74%	4,44%	4,97%	5,82%	6,42%	5,37%
Midpiece Morph.	**22,13%^{AB}**	**7,98%^{B}**	**29,12%^{A}**	**32,43%^{A}**	**30,74%^{A}**	**34,46%^{A}**	**30,33%^{A}**	**36,92%^{A}**	**23,09%^{AB}**	**39,11%^{A}**
95% Lower bound	15,64%	4,30%	17,36%	19,93%	17,92%	20,18%	18,30%	23,49%	12,21%	23,77%
95% Upper bound	29,75%	12,78%	43,91%	47,95%	47,00%	52,55%	45,39%	53,38%	37,41%	58,26%
Tail Morph.	**4,66%^{A}**	**10,59%^{A}**	**5,36%^{A}**	**8,82%^{A}**	**9,07%^{A}**	**11,26%^{A}**	**2,33%^{A}**	**10,79%^{A}**	**6,26%^{A}**	**14,34%^{A}**
95% Lower bound	2,47%	7,12%	1,84%	4,04%	3,95%	5,19%	0,32%	5,40%	2,18%	7,34%
95% Upper bound	7,53%	14,74%	10,73%	15,44%	16,28%	19,64%	6,18%	18,02%	12,43%	23,65%
Total Morph.	**32,59%^{AB}**	**25,22%^{B}**	**38,94%^{AB}**	**45,24%^{AB}**	**44,33%^{AB}**	**49,87%^{AB}**	**36,91%^{AB}**	**53,37%^{A}**	**34,16%^{AB}**	**58,85%^{A}**
95% Lower bound	25,58%	19,09%	26,74%	32,00%	30,46%	34,39%	25,06%	38,89%	22,14%	41,91%
95% Upper bound	40,46%	32,18%	53,42%	60,76%	60,81%	68,23%	51,04%	70,12%	48,78%	78,67%
Acrosome Integrity	**14,39%^{A}**	**11,49%^{AB}**	**6,87%^{AB}**	**6,39%^{AB}**	**5,87%^{AB}**	**8,39%^{AB}**	**7,89%^{AB}**	**5,22%^{AB}**	**4,54%^{AB}**	**4,09%^{B}**
95% Lower bound	10,90%	8,40%	3,30%	2,96%	2,45%	3,97%	4,01%	2,19%	1,62%	1,25%
95% Upper bound	18,36%	15,06%	11,74%	11,11%	10,78%	14,43%	13,06%	9,55%	8,94%	8,56%

^{A,B} Different superscript letters within rows denote significant differences ($P \leq 0.05$).

The cryopreservation of aspirated sperm samples had a negative impact on the survivability of the samples when compared to the same samples pre-cryopreservation (Table 5.5). Fresh sperm samples, regardless of whether sperm were aspirated immediately or exposed to prolonged storage (i.e. 24h) at 5°C, exhibited a higher survivability when compared to all of the cryopreserved samples, regardless of trehalose supplementation or thawing rate ($P \leq 0.05$; Table 5.5). It is thus evident that cryopreservation had a significant effect on the survival rate of aspirated African buffalo spermatozoa.

The fresh sperm samples that were exposed to prolonged storage at 5°C prior to aspiration exhibited a lower occurrence of morphology abnormalities when compared to samples after exposure to cryopreservation and thawing at 80°C for 5 seconds and supplemented with trehalose, regardless of the post-slaughter processing method used (25.22% vs. 53.37% and 58.85%; $P \leq 0.05$; Table 5.5).

Fresh sperm samples aspirated from the testes post-slaughter resulted in a higher percentage of intact acrosomes when compared to trehalose supplemented, fast-thawed sperm samples aspirated from testes post-storage (24h at 5°C) (14.39% vs. 4.09%; $P \leq 0.05$; Table 5.5). Thus it can be assumed that treatments thawed at 80°C for 5 seconds consisting trehalose supplementation resulted in a higher amount of morphological abnormalities as well as fewer intact acrosomes.

The paired T test conducted to determine the difference between quality parameters of fresh and cryopreserved samples showed that samples originating from testes that were aspirated without prolonged storage post-slaughter differed significantly from the non-cryopreserved treatments in terms of viability and acrosome integrity, regardless of thawing rate or trehalose supplementation. Samples originating from testes subjected to prolonged storage (i.e. 24h) at 5°C showed a decline in viability and an increase in midpiece abnormalities regardless of trehalose supplementation, and thawed at the two respective thawing rates.

Table 5.6 The influence of herd on the combined comparison between the fresh and cryopreserved aspirated spermatozoa samples from testes collected from adult bulls culled as part of a TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

Parameter	Herd A	Herd B	Herd C
Viability	**15,18%^{B}**	**21,06%^{A}**	**12,85%^{B}**
95% Lower bound	13,27%	17,28%	11,45%
95% Upper bound	17,22%	25,21%	14,33%
Head Morphology	**2,96%^{A}**	**2,78%^{A}**	**2,76%^{A}**
95% Lower bound	2,10%	1,46%	2,09%
95% Upper bound	3,95%	4,51%	3,51%
Midpiece Morphology	**27,23%^{A}**	**26,21%^{A}**	**29,87%^{A}**
95% Lower bound	21,93%	17,71%	25,41%
95% Upper bound	33,11%	36,38%	34,68%
Tail Morphology	**10,24%^{A}**	**6,79%^{A}**	**6,98%^{A}**
95% Lower bound	7,74%	3,59%	5,34%
95% Upper bound	13,09%	11,01%	8,84%
Total Morphology	**43,63%^{A}**	**37,63%^{A}**	**42,96%^{A}**
95% Lower bound	37,69%	28,54%	38,27%
95% Upper bound	49,99%	47,98%	47,93%
Acrosome Integrity	**7,59%^{AB}**	**4,79%^{B}**	**9,77%^{A}**
95% Lower bound	5,78%	2,54%	8,11%
95% Upper bound	9,64%	7,76%	11,58%

^{A,B} Different superscript letters within rows represent significant differences ($P \leq 0.05$).

Sperm samples obtained from Herd A and Herd C bulls exhibited an increased survivability when compared to those obtained from herd B bulls for the comparison of fresh and

cryopreserved samples (15.18% and 12.85% vs. 21.06%; P ≤ 0.05; Table 5.6). Samples obtained from Herd B bulls resulted in a lower occurrence of intact acrosomes when compared to those from herd C bulls (4.79% vs. 9.77%; P ≤ 0.05; Table 5.6).

5.5 Discussion

The aim of this part of the study was to determine the effect of trehalose supplementation (i.e. 0mM or 50mM trehalose) and thawing rate (i.e. thawing at 37°C for 35 seconds or 80°C for 5 seconds) on the motility, viability, acrosome integrity, and morphology (i.e. in terms of total morphology, head morphology, midpiece morphology and tail morphology) of epididymal sperm obtained from adult African buffalo spermatozoa.

When considering samples exposed to the same thawing rate and pre-aspiration processing procedures (i.e. immediate aspiration or prolonged storage at 5°C prior to aspiration) with the only difference being whether trehalose supplementation occurred or not, it is evident that the presence of trehalose has a positive impact on the motility of aspirated sperm.

Trehalose supplementation resulted in a higher survivability of cryopreserved sperm samples that were exposed to a fast thawing rate after cryopreservation and immediately aspirated post-slaughter. The production of ATP in the mitochondria of mammalian spermatozoa occurs through glycolysis that requires the availability of glucose or glycogen in order for the chemical pathway to occur for ATP to be produced (Piomboni *et al.,* 2011; Mukai & Travis, 2012; Ferramosca & Zara, 2014). Due to trehalose being a disaccharide it can act as an energy source for spermatozoa thus aiding in motility as well as survivability of sperm samples during equilibration, cryopreservation and thawing/post-thawing processes. Sperm from buffalo species (*Bubalus bubalis*) were found to have a lower oxygen uptake when compared to other species such as cattle bulls and thus a decrease in motility occurs when samples are exposed to an aerobic pathway such as oxidative phosphorylation when compared to an anaerobic pathway such as glycolysis (Vale *et al*., 2014).

Due to trehalose being a nutritive supplement as well as a membrane stabiliser, sperm viability as well as motility for the samples subjected to trehalose supplementation yielded a better result. Trehalose prevents the formation of ice crystals due to dehydration of spermatozoa and thus the viability of spermatozoa is improved when supplemented with trehalose (Shaikh *et al*., 2016. Zhu *et al*., 2017; Iqbal e*t al.* 2018). The results obtained for all treatments in terms of motility were poorer than expected. Poor motility can potentially be attributed to the duration of the time the buffaloes spent in the boma, repeated darting, disease and human presence prior to culling causing an increased level of stress to the buffaloes. During carcass evaluation of the African buffalo used during this study various

lesions caused by bovine tuberculosis infection were found. These lesions were found in areas such as the lymph nodes, lungs as well as various glands. Prolonged exposure to stressors can lead to an increase in lactate production which disrupts homeostasis (Garcia-Alvarez *et al*., 2014). Cytoplasmic acidification can occur when there are increased levels of lactate present in the blood which has an overall negative effect on spermatozoa (Matsuzaki *et al*., 2015).

Results, although not all significant, indicated that trehalose supplementation had a positive effect on the survivability of the spermatozoa, the opposite was found for the occurrence of tail abnormalities. When the percentage of abnormal tail morphology was considered, the samples that were characterised by a significantly higher percentage of tail abnormalities were all subjected to cryodiluent supplemented with trehalose. The poor tail morphology could also potentially be attributed to the stress experienced by the spermatozoa due to factors such as processing, storage, cryopreservation etc. as stress decreases the overall membrane stability of a sperm cell and thus potentially has a negative effect on the tail morphology (Coubrough, 1985; Alejandro *et al*., 2014).

Although fertilising ability was not investigated for this study, the improved viability could mean that the overall fertilising ability of the samples was also improved when exposed to trehalose (Tuncer *et al.,* 2013). However, increased fertilising ability due to trehalose supplementation would mainly benefit IVF (*in-vitro* fertilisation) purposes due to the tail morphology being negatively affected by the presence of trehalose and thus reducing the overall swimming capabilities which is necessary for successful fertilisation during AI.

To the best of our knowledge, this study presented the first ever results on the effect of trehalose supplementation on the motility, viability, morphology and acrosome integrity of African buffalo spermatozoa.

A rapid thawing rate (i.e. at 80°C for 5 seconds) led to poor survivability of aspirated sperm samples, particularly when compared to samples thawed at a traditional rate (i.e. 37°C for 35 seconds). This supports two studies that were conducted by Correa *et al*. (1996) and Shah *et al*. (2016). In both these studies it was concluded that spermatozoa responded favourably to and maintained the overall viability at a thawing rate of 30 seconds at 37°C. A temperature of 80°C would lead to the denaturation of the protein within sperm that result in a disruption of the membrane structure, which in turn negatively affects sperm viability and motility (Holme, 2019).

To the best of our knowledge there is no information on the effects that the pharmaceutical products used during this study, namely azaperone and diprenorphine, have on the reproductive capabilities and gamete quality of large herbivores.

The herds which the African buffaloes populated had an effect on the results achieved, namely for the survivability as well as the acrosome integrity of aspirated sperm samples. Despite differing parameter results between the three herds there is no indication as to why the results differed due to all management practices, to the best of our knowledge, being identical (all herds were in a 10km radius from one another) as well as the total time that the buffalo herds were within the boma not having a significant effect on the results obtained. A plausible reason could have been the gender composition of the herd however the bulls only had to service 2.34, 3.67 and 3.8 cows in herds A, B and C respectively. Mature African buffalo bulls (*Syncerus caffer*) that can potentially contribute to the genetic pool usually make up 15% of the herd (Hildebrandt, 2014). This equates to one bull servicing approximately 6.67 cows, and thus the sex ratios seen in the herd composition for this study would not have had a negative impact on the fertility and fecundity of the population.

From the comparison of non-cryopreserved and cryopreserved samples it is evident that cryopreservation significantly reduced the viability of spermatozoa regardless of thawing rates used or supplementation of the cryodiluent. This supports the findings of Lambrechts *et al.* (1999) which found that cryopreservation and thawing had a significant effect on the viability of epididymal African buffalo spermatozoa.

5.6 Conclusion

Trehalose supplementation had a positive effect on the viability as well as motility of cryopreserved African buffalo spermatozoa samples however; the tail morphology was negatively affected by the addition of trehalose to Triladyl®. A fast thawing rate of 80°C for 5 seconds is not recommended when thawing African buffalo epididymal spermatozoa due to a significant decrease in the overall sperm viability. Although it was found that herd had a significant effect on some of the sperm parameters, it is difficult to relate these differences to a specific herd effect, for the herds were similar in terms of age and sex, were captured from the same region, and duration of time spent in the boma was also similar for the herds. Recommendations for future studies include investigating the effect of differing concentrations of trehalose and the effect of trehalose over time during the equilibration period on the motility, viability, morphology, acrosome integrity and fertilising abilities of epididymal African buffalo spermatozoa. Methods to measure the presence and effect of lactate should also be considered to determine to what extent stress affects the quality of spermatozoa. The potential benefit and effect of antioxidant supplementation to aspirated

sperm samples should be considered in terms of the motility, viability, morphology, acrosome integrity and fertilising ability.

5.7 References

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Chapter 6

The potential of flow cytometry to evaluate the post-thaw viability of epididymal African buffalo (*Syncerus caffer*) spermatozoa

6.1 Abstract

This study investigated the use of flow cytometry to quantify the post-thaw viability of epididymal African buffalo (*Syncerus caffer*) spermatozoa. The sperm samples were obtained from adult African buffalo testes post-slaughter (exposed to 0h of intact storage at 5°C) and from testes stored intact at 5°C for 24h. In addition to this treatment, the epididymal sperm samples were subjected to cryopreservation in a cryodiluent supplemented with 0mM and 50mM trehalose. Cryopreserved samples were thawed at 37°C for 35sec, and were then stained with propidium iodide and SYTO 9 nucleic acid for flow cytometry analysis. The objective flow cytometry analysis method yielded higher average percentage viability, when compared to that obtained with the subjective nigrosine-eosin viability stain method. A correlation matrix and linear regression analysis were performed to determine whether the flow cytometry analysis can replace the nigrosine-eosin vitality stain method in the assessment of sperm vitality assessment in sperm evaluation protocols to potentially predict acrosome integrity. No relationship between the two parameters was found. Findings from this study will assist in the development of a flow cytometry analysis method to assess African buffalo sperm viability and acrosome integrity in future studies.

Keywords: Trehalose, African buffalo, flow cytometry, epididymal, spermatozoa.

6.2 Introduction

The African buffalo (*Syncerus caffer*) is a member of the so-called Big 5 and is highly sought after for both eco-tourism as well as trophy hunting operations. In 2015, 12% of the total hunting activity earnings, which equates to R145 million to South Africa's annual foreign exchange currency, was earned due to the hunting of African buffaloes (Coleman, 2018). The African buffalo plays an important role in the ecosystem due to this species being bulk grazers and thus maintaining the grazing quality by utilising the long, fibrous grasses thus converting the grazing to short grasslands which is more suitable for smaller herbivores such as the impala (*Aepyceros melampus*) (Michel & Bengis, 2012, Krugerpark.co.za, 2017). The African buffalo is susceptible to four economically significant diseases namely foot and

mouth disease, Corridor disease, Bovine tuberculosis and Brucellosis (Laubscher & Hoffman, 2012) and these diseases have affected the buffalo populations in the Kruger National Park in Mpumulanga as well as Hluhluwe-iMfolozi in Kwa-Zulu Natal (De Vos & Van Niekerk, 1969; Laubscher & Hoffman, 2012). Due to the impact of these diseases on the production and reproduction of the African buffalo, ad seen against the background that this species numbers have declined in the last few decades, collection and processing protocols for spermatozoa and oocytes of this species needs to be designed and/or refined to ensure that samples with a good viability and fertilizing ability are processed and stored in cryopreserved form in a genome resource bank (GRB) for this species.

In standard sperm evaluation protocols, the nigrosine-eosin (NE) vitality stain is a popular method to use to evaluate viability of ejaculated and epididymal spermatozoa, for it is a cost-effective and time-efficient method to use (Williams & Pollak; 1950). One limitation of this technique, however, is that it yields subjective results. Even though subjective, NE analysis still provides an indication of the potential of a sample to be used for GRB purposes. Cryopreserved African buffalo sperm samples stored in a GRB can be used for research purposes, for the AI of live cows or used in the production of African buffalo embryos using a species-specific IVEP protocol. To obtain as close as possible indication of the correlation between laboratory- and field fertility and to standardize between andrology laboratories, the use of an objective method such as flow cytometry assessment of vitality potentially presents an approach to address the subjective nature of the NE method.

Flow cytometry provides an objective approach using various fluorescent dyes that can be used to quantify spermatozoa quality parameters which includes, but is not limited to viability, acrosome integrity, and DNA integrity (Cordelli *et al*., 2005). During analysis, stained cells suspended in a solution move at a rate of at least 10 000 cells per second passed lasers, thus providing rapid and sensitive quantification of sample populations (ThermoFisher Scientific, not dated.). The scattered light emitted by the lasers is filtered by mirrors for signal amplification once the light has reached the photodetectors. For viability analysis, a common dye/probe used is propidium iodide due to it being membrane impermeable. Due to this characteristic the dye will only enter damaged cells and thus only these cells will emit a red fluorescence (Martinez-Pastor *et al*., 2010). To the best of our knowledge, this study presents the first information on the use of flow cytometry for the analysis of African buffalo sperm viability and acrosome integrity.

The aim of this research was to determine whether flow cytometry would be a useful method for African buffalo sperm analysis. This includes the comparison between subjective (nigrosine-eosin dye) and objective (flow cytometry) viability evaluation of epididymal African

buffalo spermatozoa. This was carried out to determine whether flow cytometry is a viable method to use for sperm analysis.

6.3 Methodology

6.3.1 Experimental location

The collection of samples took place in the Hluhluwe-iMfolozi game reserve during the 2018 Bovine Tuberculosis monitoring operation. The reserve is located in the northern part of the Kwa-Zulu Natal Province, and is characterized by a humid, subtropical climate. This region experiences hot, wet summers and mild, dry winters, with an average annual temperature of 19.5°C and an average annual rainfall of 957mm.

Post-thaw evaluation was carried out in the Animal Physiology laboratory of the Department of Animal Sciences of Stellenbosch University, Stellenbosch, Western Cape. This area is characterized by a Mediterranean climate and experiences cool, wet winters and hot, dry summers. This area is characterised by an average annual temperature of 16.4°C and an annual rainfall of 802mm (Climate-data.org, 2019).

6.3.2 Experimental animals and collection of testes

Testes were collected from African buffalo (Syncerus caffer, N=114) that were culled using a rifle shot as part of a Bovine Tuberculosis monitoring program (with animals testing positive for bovine tuberculosis being eradicated) carried out in the Hluhluwe-iMfolozi Game Reserve, animals culled consisted of both sexes. Testes were collected from 26 adolescent and mature bulls with 21 mature bulls contributing to this study, due to the adolescent males not producing functioning sperm. Three individual herds (i.e. Herd A, Herd B and Herd C) that consisted of adults, young adults, sub adults, juveniles and calves of both sexes, were culled in the period 17/07/2018 till 02/08/2018. Herds A and B were captured in the same location of the Reserve, and Herd C was captured 8.2km away from this location. Prior to culling the animals were maintained in a boma to facilitate sedation for blood sampling and skin tests to determine the bovine tuberculosis status as well as the extent of infection.

The pharmaceutical products used to achieve sedation included Stresnil (azaperone-M99, Sigma-Aldrich) for sedation as well as M5050 (diprenorphine (Sigma-Aldrich) to reverse the sedation. Care was taken to ensure that all animals had *ad libitum* access to feed and fresh drinking water during the holding period. Herds A, B and C were maintained in the boma between 16 to 25 days, 15 to 22 days and 10 to 19 days, respectively.

6.3.3 Experimental design

The experimental design for experiment 4 was a 2x2 factorial design. The first value of the treatment label indicates whether the testis was processed immediately after slaughter (i.e. 0 hours intact) or whether the testis was subjected to intact storage at 5°C for 24 hours prior to processing (i.e. 24 hours intact). The 37 in the treatment labels refers to the thawing temperature of the cryopreserved samples (i.e. 37°C). The third section of the treatment labels symbolises whether the spermatozoa were cryopreserved in just the cryodiluent (namely Triladyl®) or whether the Triladyl® was supplemented with trehalose (i.e. T+T). With the comparison of subjective and objective evaluation methods (section 6.4.5) data labels LM (Light Microscopy) equate to the subjective analysis whereas FC (Flow Cytometry) equates to the objective analysis of the sample viability.

6.3.4 Processing of testes

For allocation to the respective treatment groups, the two testes of each bull were collected, and from each set, one testis was processed immediately after culling, whereas the other testis was stored intact and protected against dehydration, at 5°C for 24h before being processed.

Processing of each testes and aspirated sperm samples were carried out at room temperature (about 22°C).The *tunica albuginea* of each testis was cut open to expose the testis and epididymis. After removal of the *tunica albuginea,* testis length, width and circumference were recorded. The cauda epididymis including a part of the *vas deferens* was then detached from the testis, and transferred to an unused 90mm Petri dish, and care was taken to prevent dehydration of the epididymis.

To obtain the epididymal sperm samples, the tubules of each cauda epididymis were exposed by removing the connective tissue and blood vessels using a scalpel blade (size no. 11, lasec) and a pincette. After exposure, a large portion of the tubules before transition into the vas deferens was removed and transferred to a 65mm Petri dish containing 3mL Ham's-F10 (Sigma-Aldrich) prewarmed to 37°C (Lambrechts *et al*., 1999; Herold *et al*., 2006). A slicing method was used to allow for the release of the spermatozoa into the collection medium (Bertol, 2016). Once slicing was completed the tubules were rinsed with another 1mL Ham's F-10 (pre-warmed to 37°C), to ensure optimal sperm recovery. The Petri dish containing the epididymal spermatozoa and Ham's F-10 nutrient combination was then placed on a warming plate (MH6616, Electrothermal) set to 37°C in order to prevent temperature fluctuations.

6.3.5 Dilution of samples

The amount of straws of epididymal spermatozoa that would be cryopreserved was dependent on the concentration as well as the volume of overall sample available. The epididymal spermatozoa and Ham's F-10 nutrient-rich mixture was subjected to two different treatments prior to cryopreservation, namely dilution to a concentration of 30 million epididymal spermatozoa per millilitre using Triladyl® (Lambrechts *et al*., 1999; Herold *et al*., 2006) or dilution to a concentration of 30 million epididymal spermatozoa per millilitre using Triladyl® containing 50mM of Trehalose (stock solution of Trehalose was 100mM).

- $n\ of\ straws\ desired\ x\ 0.25ml = total\ v\ needed\ (in\ ml)$
- $\frac{desired\ spermatozoa\ concentration}{actual\ spermatozoa\ concentration} = v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v$
- $v\ in\ ml\ of\ spermatozoa\ mixture\ per\ mL\ of\ total\ v\ x\ total\ ml\ needed = total\ v\ of\ spermatozoa\ mixture\ needed$
- $Total\ v\ needed - total\ v\ of\ spermatozoa\ mixture\ needed = v\ of\ cryodiluent\ needed$

For more information on the dilution of samples please see section 3.5.5.1.

6.3.6 Cryopreservation and equilibration of spermatozoa

Once the epididymal spermatozoa and Ham's F-10 nutrient-rich mixture was diluted with the respective pre-warmed (37°C in a waterbath) cryodiluent (see 5.3.5) (after gradual addition via pipette in order to prevent chemical shock to the epididymal spermatozoa) the 15 mL falcon tubes were everted 2-3 times and placed in a 250ml glass beaker containing H_2O and then equilibrated at 5°C for approximately 3 to 4 hours (Lambrechts *et al*., 1999; Herold *et al*., 2006). At this point the 0.25cc French straws that would be used for the cryopreservation of the spermatozoa samples were marked clearly (information included animal ID, date, sample treatment) and placed in the refrigerator at 5°C in order to reduce the risk of temperature shock to the spermatozoa once the loading of the French straws commenced. After the 3-4 hours of equilibration the spermatozoa/cryodiluent mixtures were loaded into clearly marked 0.25cc French straws. The French straws' end without the cotton wool plug was then sealed off using a PVC powder. Once the French straws were loaded and sealed they were placed in Liquid Nitrogen vapour (-140 to -180°C) for 15 minutes and then submerged into the liquid nitrogen (at -196°C) for 5 minutes (Herold *et al*., 2006; Swelum *et*

al., 2011) before placing them into the canes and goblets of the liquid nitrogen cryopreservation tank.

6.3.7 Flow cytometry analysis

A stain was prepared by mixing SYTO 9 nucleic acid stain and Propidium iodide (Molecular probes; L34856) at a 1:1 ratio, and placed in a centrifuge to mix (200rpm, 3 seconds). Cryopreserved African buffalo spermatozoa straws with a volume of 0.25cc (for cryopreservation protocol refer to section 3.5.5) were thawed for 35 seconds in a water bath maintained at 37°C to ensure no temperature fluctuations occurred during thawing (Swelum *et al*., 2011). After thawing the straw was dried, and cut at one end to release the straw content into a 1ml Eppendorf tube (Lasec, South Africa). The samples were transferred to a 2000µL Eppendorf tube and transported to the fluorescence laboratory in the Department of Physiological Sciences, Stellenbosch University in Stellenbosch, Western Cape. 300µL of thawed epididymal spermatozoa sample was transferred via pipette to a clearly marked (ID and Treatment) Falcon tube compatible with the BD FACSMelody machine (Becton Dickinson, San Jose, USA). 0.6µL of dye mixture was added to the epididymal spermatozoa samples using a pipette. The sperm-dye mixture was lightly mixed and allowed to stand for 10 minutes in order for the dye to saturate the spermatozoa. The tubes were then placed on a vortex for 1-2 seconds before being placed in to the BD FACSMelody machine for analysis and sample recording. Analysis of the samples was performed by the machine compatible computer program, BD FACSChorus. General analysis included the creation of scattergrams based on the analysed samples. Samples that fluoresced green indicated spermatozoa with intact cell membranes (blue on scattergrams depicted in section 6.4.2) and thus classified as live spermatozoa whereas spermatozoa with damaged membrane, thus dead spermatozoa, fluoresced far less and could even show a red fluorescence (red on scattergrams depicted in section 6.4.2).

For the sake of this study, the flow cytometry results are represented as different populations with the live, dead and intermediate sub-populations being shown in the scattergraphs (section 6.4.2). The perCP seen on the Y-axis of the graph provides a multi-color analysis tool which allows various subpopulations of spermatozoa, dependent on their viability, to be visualised. The SSC (Side Scatter) seen on the Y-axis of the graph provides information on the internal structure of the spermatozoa and is measured by the deflection of the laser on the intracellular structures of the cell.

6.3.8 Statistical analysis

The statistical program used to analyse the data collected was Microsoft XLStat and it was found that the data was normally distributed and the residuals of the data were homoscedastic. Nigrosine-eosin results for the fresh samples and the data for the viability analysis using flow cytometry were compared using a one-way ANOVA to determine whether there was a significant difference between the two analysis methods. Linear regression and correlation tests were carried out to determine whether the acrosome integrity achieved during Nigrosine-eosin analysis could be linked to the viability results achieved during flow cytometry.

6.4 Results

6.4.1 Descriptive statistics

Table 6.1 represents the descriptive statistics for the respective sperm parameters recorded during the study. The largest degree of variation was recorded for the % live spermatozoa (0.557).

Table 6.1 The average (mean ± SD) results recorded for the various parameters included in the study flow cytometry samples collected from African buffalo bulls during a bovine tuberculosis monitoring operation in the Hluhluwe-iMfolozi Game Reserve in 2018.

Parameter	Mean ± SD	Range	Coefficient of Variation
% Live	29.40% ± 16.39%	0.44% - 57.48%	0.557
% Dead	67.76% ± 14.94%	41.19% - 98.68%	0.220

6.4.2 Flow cytometry scatter graphs

The graphs produced by the BDFACSChorus program indicated three different subpopulations, namely a live, dead and an intermediate cluster. The figures presented below belongs to one African buffalo bull however, the treatments differ.

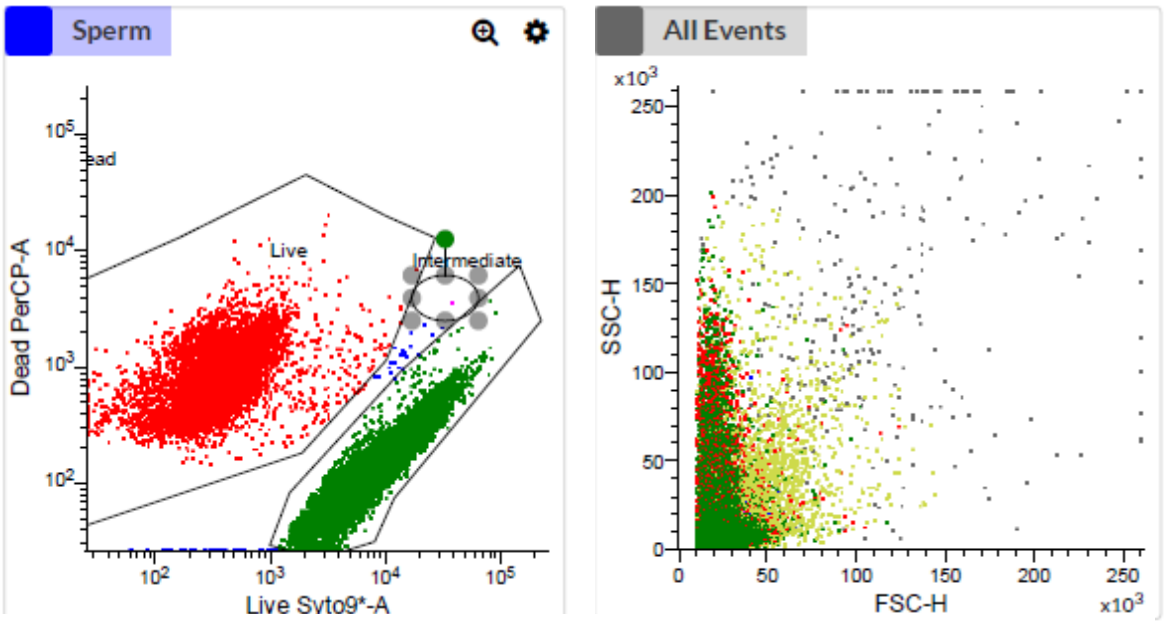

Figure 6.1 Visual representation of the 3 subpopulations created during sperm viability analysis via flow cytometry (Buffalo ID and Treatment: B58 0h_37_Tri, % Live = 45.86%). The three subpopulations include live sperm, dead sperm and intermediate (undetermined viability state).

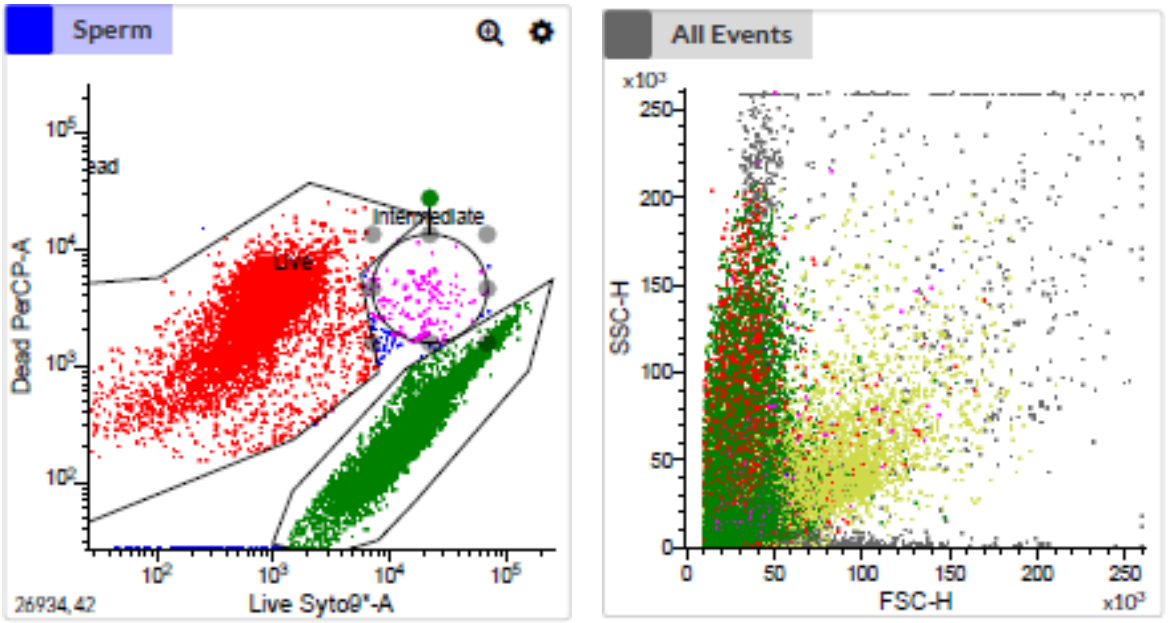

Figure 6.2 Visual representation of the 3 subpopulations created during sperm viability analysis via flow cytometry (Buffalo ID and Treatment: B58 0h_37_T+T, % Live = 57.48%). The three subpopulations include live sperm, dead sperm and intermediate (undetermined viability state).

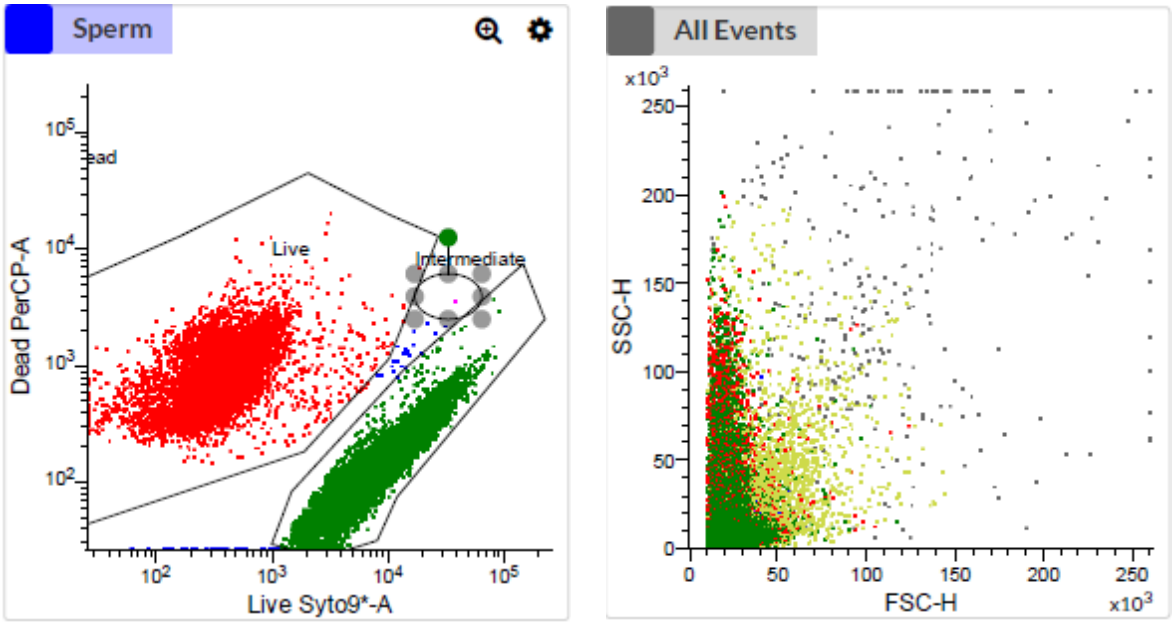

Figure 6.3 Visual representation of the 3 subpopulations created during sperm viability analysis via flow cytometry (Buffalo ID and Treatment: B58 24h_37_Tri, % Live = 35.06%). The three subpopulations include live sperm, dead sperm and intermediate (undetermined viability state).

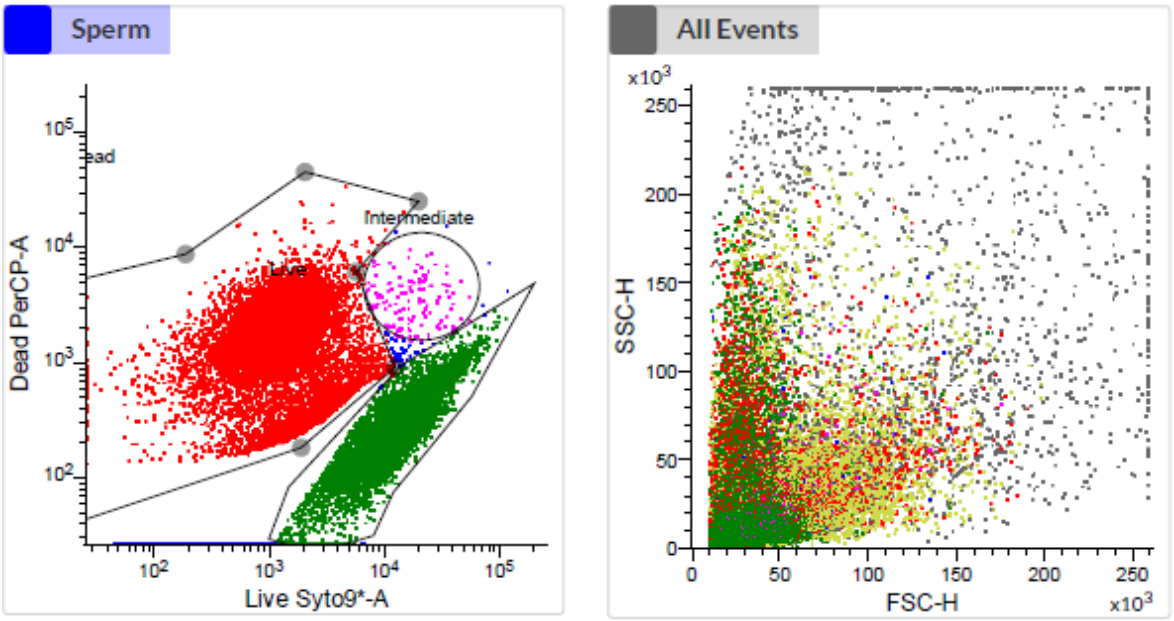

Figure 6.4 Visual representation of the 3 subpopulations created during sperm viability analysis via flow cytometry (Buffalo ID and Treatment: B58 24h_37_T+T, % Live = 42.14%). The three subpopulations include live sperm, dead sperm and intermediate (undetermined viability state).

6.4.3 Comparison of Flow Cytometry with Nigrosine-Eosin subjective analysis

The viability data achieved from analysing cryopreserved samples, which was analysed via subjective nigrosine-eosin staining, was compared to the objective method of flow cytometry (Table 6.2).

Table 6.2 The comparison of the objective analysis of viability and subjective analysis of viability of epididymal African buffalo spermatozoa collected from adult bulls culled as part of the TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

Treatment	LS Means ± SEM
0h_37_T+T_FC	32,87%^{A} ± 3,67%
0h_37_T+T_LM	17,15%^{ABC} ± 3,82%
0h_37_Tri_FC	28,79%^{AB} ± 3,67%
0h_37_Tri_LM	12,35%^{BC} ± 3,82%
24h_37_T+T_FC	31,96%^{A} ± 4,68%
24h_37_T+T_LM	10,79%^{BC} ± 5,01%
24h_37_Tri_FC	22,97%^{ABC} ± 4,42%
24h_37_Tri_LM	8,37%^{C} ± 4,68%

^{A,B} Different superscript letters within rows denote significant differences (P ≤ 0.05)

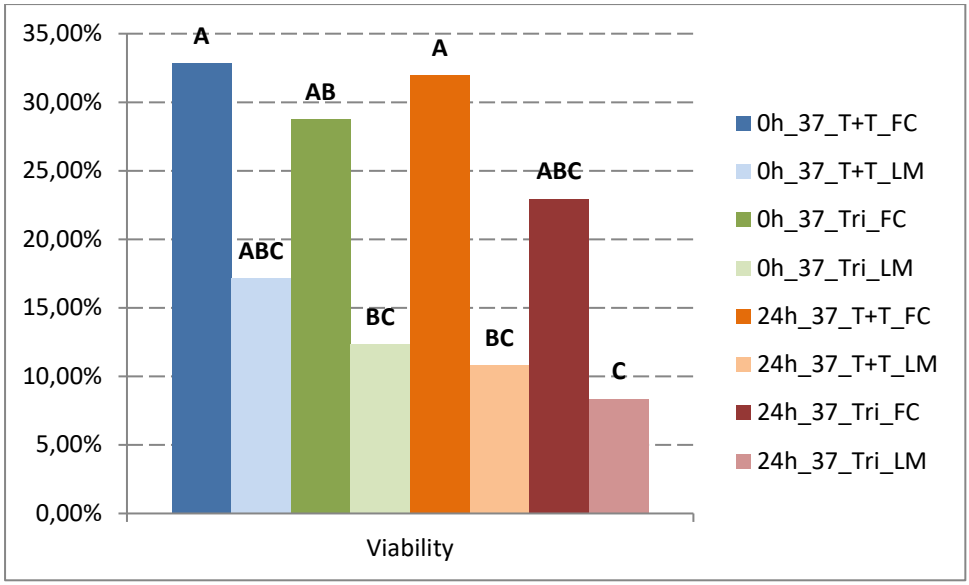

Figure 6.5 The comparison of the objective analysis of viability and subjective analysis of viability of epididymal African buffalo spermatozoa collected from adult bulls culled as part of the TB monitoring operation during 2018 in the Hluhluwe-iMfolozi Game Reserve.

When comparing the same treatments with one another the objective analysis (i.e. flow cytometry) of the samples that were processed after prolonged storage (i.e. 24h) of the testis at 5°C prior to spermatozoa aspiration and were subject to trehalose supplementation had a higher survivability when compared to samples analysed subjectively via light microscopy (31.96% vs. 10.79%; $P \leq 0.05$; Table 6.2). When considering the samples exposed to the same analysis method (i.e. flow cytometry or light microscopy) there were no differences observed between the 4 different treatments ($P > 0.05$; Table 6.2).

A correlation matrix and linear regression analysis was carried out to determine whether there was a relationship between the acrosome integrity data obtained via Nigrosine-eosin analysis and the viability of sperm samples obtained via flow cytometry analysis (Figure 6.6).

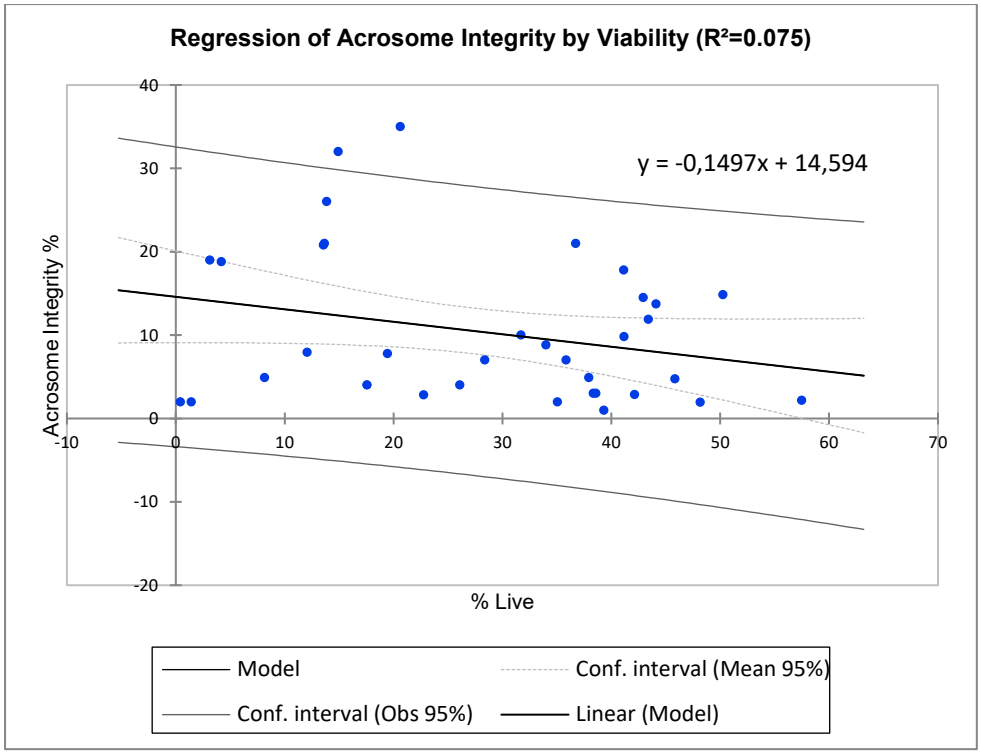

Figure 6.6 Graphical presentation of the linear regression carried out on the acrosome integrity data obtained via nigrosine-eosin analysis versus the viability data obtained from flow cytometry analysis.

It is evident that there is no relationship between the acrosome integrity data obtained via Nigrosine-eosin analysis and the viability of sperm samples obtained via flow cytometry analysis.

6.5 Discussion

The aim of the study was to determine the potential of using flow cytometry for the objective use of spermatozoa analysis. Flow cytometry is an efficient and sensitive way of recording sperm viability and appears to have improved accuracy when compared to Nigrosine-Eosin stain counting which has limitations and human error due to being a subjective method (Cordelli *et al*., 2005). To the best of our knowledge, there is no literature reviewing the use of flow cytometry for the analysis of African buffalo spermatozoa quality parameters.

Conventional sperm analysis methods only consider a small portion of the population and thus the results are less accurate when compared to flow cytometry which utilises a much larger population. Flow cytometry has been used for the analysis of various sperm-related research trials which include sperm obtained from boar (*Sus domesticus*) (Torres *et al*., 2016), Nili-Ravi buffalo (*Bubalus bubalis*) sperm for the analysis of ROS (Li *et al*., 2012), acrosome and membrane integrity assessment in the Asian elephant (*Elephas maximus*)

(Thongtip *et al.,* 2004), and to determine the concentration and plasma membrane integrity of Zebrafish (*Danio rerio*) spermatozoa (Yang *et al*., 2016).

In a study conducted by Battut *et al*. (2017), the prediction of the fertility of stallion (*Equus caballus*) frozen-thawed semen was investigated using Computer-Assisted Motility analysis (CASA), microscopic analysis as well as flow cytometry. In their study it was concluded that a combination of these methods could be used to provide a higher accuracy of prediction than when only CASA is used.

The acrosome integrity of sperm samples during flow cytometry in this study was not measured and it was also found that there was no relationship between the acrosome integrity of sperm samples analysed via nigrosine-eosin analysis and the viability obtained from flow cytometry. Thus, it was not possible to predict the possible acrosome integrity of the samples. However, the effect of flow cytometry procedures on the acrosome integrity of sperm samples needs to be considered. In a study by Len *et al.* (2008), it was found that a centrifugal force up to 900g did not have a negative effect on the progressive motility, viability or acrosome integrity of equine (*Equus caballus*) spermatozoa. Way *et al*. (1994) found that a dye containing propidium iodide and *Pisum sativum* agglutinin resulted in an accurate analysis of acrosome integrity and viability of bovine spermatozoa.

The flow cytometry method indicated three subpopulations namely live, intermediate and dead. The intermediate subpopulation represents spermatozoa that are potentially undergoing apoptosis and thus do not fall into the other two subpopulations. This study provided evidence that the use of flow cytometry can provide a more accurate indication of African buffalo epididymal sperm viability, when compared to more subjective methods such as the NE method. However, the data available for acrosome integrity from NE analysis was not associated with the flow cytometry viability results.

6.6 Conclusion

Flow cytometry is a method that can be used to improve the accuracy of spermatozoa analysis. In this study, the flow cytometry method provided an indication of three distinct subpopulations in terms of viability, i.e. a live, dead and intermediate cluster. The intermediate cluster represents spermatozoa that are potentially in the process of undergoing apoptosis. Further studies need to investigate this.

Acrosome data obtained with the NE analysis was not related to the flow cytometry results obtained in this study. Future studies need to investigate the use of different dyes to develop

a species-specific flow cytometry protocol for the assessment of acrosome integrity, morphology, and concentration for wildlife species such as the African buffalo.

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Chapter 7

General conclusion and recommendations

The African buffalo (*Syncerus caffer*) is a member of the so-called Big 5 and contributes to the overall GDP of a country through ecotourism, trophy hunting, breeding of game as well as game products such as horns, meat, and hides. Despite the breeding efforts in industry, African buffalo populations are decreasing in size, and this species is currently listed as Near Threatened on the 2018 Global IUCN Red List with a population of approximately 400 000 individuals. This decreasing in population size can be ascribed to anthropogenic influences such as hunting and habitat decrease, and the susceptibility of African buffalo to diseases such as FMD, bovine tuberculosis, brucellosis, and corridor disease. These diseases all contribute negatively to the economy due to the zoonotic nature of some of the diseases, as well as the potential spreading to domestic cattle populations. Thus, conservation of this species is becoming increasingly important and can be done via the cryopreservation of genetic material.

The cryopreservation of genetic material not only allows for national exchange of genetics but also extends to the international exchange of genetic material to aid in conservation of species that are at risk. Due to these reasons as well as the African Buffalo being affected by various diseases it is becoming crucial to research ways in which we can use ARTs to harvest, process and store the spermatozoa from African buffalo bulls to aid in the production of offspring as well as to create stock of African buffalo genetics in the national GRB.

Assisted reproductive techniques (ARTs) form an integral component of the establishment and maintenance of GRBs. The collection or harvesting, evaluation, processing and storage of spermatozoa and oocytes of wildlife species requires the development of species-specific protocols to ensure gamete viability and fertilising ability are maintained. The process that will allow short- and long-term storage of epididymal African buffalo spermatozoa needs to be investigated and refined for potential contribution to the conservation of this species. Short-term storage eliminates the need for the cryo-storage of such samples, thus making it a less costly procedure when compared to long-term storage such as cryopreservation; however, cryopreservation allows for the long term and indefinite storage of gametes as long as the liquid nitrogen levels in the holding tank are maintained. In addition to investigating the methods of storage of gametes the various analysis procedures needs to be investigated as well in order to develop a method that allows for the objective and efficient analysis of spermatozoa parameters.

7.1 Influence of harvesting method on African buffalo (*Syncerus caffer*) spermatozoa viability and survivability

The aim of this part of the study was to determine the influence of harvesting method and duration of liquid cold storage (i.e. at 5°C) on the mass motility, viability, morphology and acrosome integrity of African buffalo spermatozoa obtained from testes collected from bulls culled as part of a TB monitoring program. The liquid storage of aspirated sperm samples at 5°C had a negative effect on the viability, midpiece morphology and total morphology, when compared to samples that were analysed immediately post-aspiration, and is thus not recommended. Storage of testes intact for 24h at 5°C had a significant negative effect on the tail morphology of the samples. Should a form of storage need to be decided on based on these results, the prolonged storage of testes intact at 5°C is recommended, motivated by that the testis-epididymal environment is more stable in terms of maintenance of sperm viability and integrity, thus minimising the deleterious effect of liquid storage for extended periods, e.g. up to 24h.

Harvesting method and liquid storage did not have a significant effect on the head morphology or acrosome integrity of the sperm samples. Findings from this study will assist in the refinement of post-mortem harvesting and processing protocols for the long term conservation of germplasm from African buffalo bulls, and also for potential use in assisted reproductive techniques to restore populations.

Future studies should investigate extending the prolonged cold storage time (e.g. up to 72h) of testes and aspirated sperm samples to determine for how long the viability and integrity of epididymal spermatozoa can be maintained post-mortem. This information is especially important to consider when processing and evaluation facilities are not readily available when animals of high genetic merit that occur in remote areas, pass away and the genetic material of these animals need to be harvested for GRB purposes. The determination of fertilising ability through methods such as a perivitelline membrane binding assay can also be considered, once it is adapted for field trials. The effect of stress caused by repeated darting, human presence, boma capture etc., on the various sperm parameters should be considered and can possibly be analysed through lactate measurements in the blood. The effect of pharmaceutical products such as M5050 and M99 on the viability and integrity of African buffalo epididymal spermatozoa need to be determined, as well as the influence of spermatogenesis studied when these products are administered repeatedly.

7.2 Influence of Trehalose and thawing rate on the post-thaw viability and survivability of African buffalo (*Syncerus caffer*) spermatozoa

The aim of this study was to determine the influence of trehalose supplementation (50mM) and thawing rate (at 37°C for 35 seconds and 80°C for 5 seconds) on the mass motility, survivability, morphology and acrosome integrity of cryopreserved African buffalo spermatozoa obtained from testes collected from bulls culled as part of a TB monitoring program. The inclusion of trehalose as part of the cryodiluent had a positive effect on the motility as well as viability of frozen-thawed sperm samples; however, trehalose supplementation negatively affected tail morphology. A fast thawing rate is not recommended due to a significant decrease in the viability of samples. The head morphology, midpiece morphology, total morphology (combination of head, midpiece and tail morphology) and acrosome integrity were not affected by the thawing rates or trehalose supplementation.

Recommendations for future studies include using differing concentrations of trehalose (e.g. 25mM, 50mM, 75mM etc.) and monitoring the effect over time during the equilibration period as well as post-thaw.

7.3 The potential of flow cytometry to evaluate the post-thaw viability of epididymal African buffalo (*Syncerus caffer*) spermatozoa

This study investigated the use of flow cytometry to quantify the post-thaw viability of epididymal African buffalo (*Syncerus caffer*) spermatozoa. The viability results of the objective flow cytometry analysis yielded higher viability values when compared to the subjective NE analysis. A correlation matrix and linear regression were carried out to determine whether the acrosome integrity results obtained from Nigrosine-Eosin analysis could be linked to the viability results obtained via flow cytometry in order to potentially predict acrosome integrity. However, no relationship between the two parameters was found. Flow cytometry is not only limited to the viability of samples but can be utilised for the analysis of acrosome integrity, morphology, count (i.e. concentration) etc.

Recommendations for future studies would be to include a wider range of parameters to be analysed via flow cytometry such as concentration, morphology and acrosome integrity.

To conclude, findings from this study contribute to the existing body of knowledge on the use of ARTs in African buffalo reproductive management and conservation. Future studies to develop flow cytometry parameters, and refinement of the existing protocols by studying the behaviour of African buffalo sperm under different experimental conditions, will improve our

understanding of factors determining African buffalo sperm resilience to processing- and cryopreservation-associated stress.

Appendix A

Table A.1 Summary of the coefficients of determination for the non-cryopreserved samples (Chapter 4). These values indicate how much of the variation in variable Y can be explained by the variation in variable X.

Parameter	R^2
Viability	0,523
Head abnormalities	0,126
Midpiece abnormalities	0,339
Tail abnormalities	0,320
Total abnormalities	0,310
Acrosome Integrity	0,068

TableA.2 Summary of the coefficient of determination for the cryopreserved spermatozoa samples (Chapter 5). These values show how much of the variation in Y can be explained by X.

Parameter	R^2
Viability	0,591
Head morphology	0,075
Midpiece morphology	0,113
Tail morphology	0,383
Total morphology	0,200
Acrosome integrity	0,144

Table A.3 Summary of the coefficient of determination for the fresh and cryopreserved spermatozoa sample from the combined data (Chapter 5). These values show how much of the variation in Y can be explained by X.

Parameter	**R^2**
Viability	0,963
Head morphology	0,111
Midpiece morphology	0,449
Tail morphology	0,392
Total Morphology	0,374
Acrosome Integrity	0,342

Appendix B

Table B.1 The data sheet used for data collection during the trial in Hluhluwe-iMfolozi Game Reserve during a TB eradication program in 2018.

Animal ID	

Parameter	**Left**	**Right**
Slaughter date		
Slaughter start		
Slaughter end		
Process start		
Process end		
Equil start		
Cryo		
Testis L (mm)		
Testis W (mm)		
Testis Circumf (mm)		
Epid W (mm)		
Epid L (mm)		
0h Time		
24h Time		
Remarks:		

Appendix C

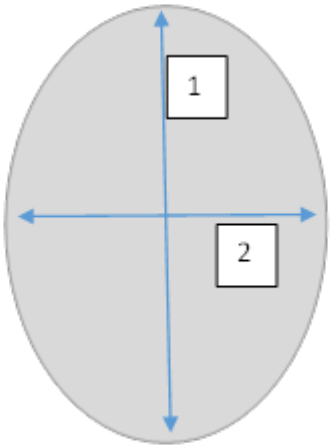

Figure C.1 Graphical depiction of how the testis measurements were taken with 1 representing the length, 2 representing the width and 3 representing the circumference.

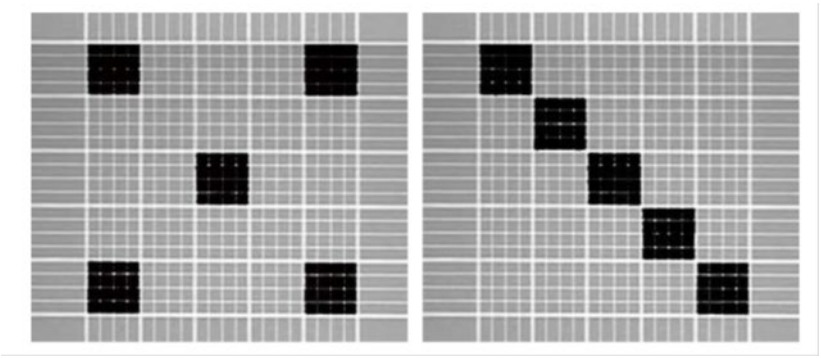

Figure C.2 Graphical depiction of the counting chambers in the McMaster slide for concentration determination of the spermatozoa samples.